NTP REPORT ON CARCINOGENS BACKGROUND DOCUMENT for PHENOLPHTHALEIN

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TABLE OF CONTENTS

NTP Report on Carcinogens Listing for Phenolphthalein 1
Listing Criteria from the Report on Carcinogens, Eighth Edition 2

1.0 CHEMICAL PROPERTIES 3
1.1 Chemical Identification 3
1.2 Physical-Chemical Properties 4

2.0 HUMAN EXPOSURE 5
2.1 Use 5
2.2 Producers, Production Process, and Production Volume 6
2.3 Environmental Exposure 6
2.4 Occupational Exposure 6
2.5 Regulations and Criteria 6

3.0 HUMAN STUDIES 9
Table 3-1. Human Studies of Effects of Exposure to Phenolphthalein 11

4.0 EXPERIMENTAL CARCINOGENICITY 13
4.1 Rats 13
4.2 Mice 13
Table 4-1 Experimental Carcinogenicity of Phenolphthalein Published Post NTP (1996) 15

5.0 GENOTOXICITY 16
5.1 Studies Summarized in NTP (1996) 16
5.2 Studies Published Post NTP (1996) 16
Table 5-1: Summary of Genotoxicity Studies Published Post NTP (1996) 18

6.0 OTHER RELEVANT STUDIES 20
6.1 Absorption, Distribution, Metabolism, and Excretion 20
6.1.1 Absorption and Metabolism 20
6.1.2 Distribution 20
6.1.3 Excretion 21

6.2 Pharmacokinetics 21
6.3 Structure-Activity Relationships 21
6.3.1 Diphenylamine Structural Analogs 21
6.3.2 Alkylating Substructure 22
6.3.3 Physical-Chemical Properties 22

7.0 MECHANISMS OF CARCINOGENESIS 22
7.1 Ovarian Cancer 22
7.2 Radical Formation 22
7.3 Genetic Toxicity 23
7.4 Thymic Lymphoma and Estrogenic Effects 23

8.0 REFERENCES 25

APPENDIX A - Excerpt from NTP Technical Report Toxicology and Carcinogenesis Studies of Phenolphthalein in F344/N Rats and B6C3F$_1$ Mice (Feed Studies), pp. 4-38, 71-91, November 1996 ..... A-1

APPENDIX B - Description of Online Searches for Phenolphthalein B-1

APPENDIX C - Report on Carcinogens (RoC), 9th Edition Review Summary C-1

NTP Report on Carcinogens Listing for Phenolphthalein

Carcinogenicity

Phenolphthalein is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of increased incidence of malignant and/or combination of malignant and benign tumors in multiple tissue sites and in multiple species. In a two-year $B6C3F_1$ mouse carcinogenicity study, NTP (1996) concluded that phenolphthalein, administered in feed, induced significant increases in the incidence of histiocytic sarcoma and lymphomas of thymic origin in males and females and malignant lymphoma (all types) and benign ovarian sex cord stromal tumors in females. In the corresponding Fischer 344 rat dietary carcinogenicity study, phenolphthalein induced significant increases in the incidence of benign pheochromocytoma of the adrenal medulla in males and females and renal tubule adenoma in males (NTP, 1996). In a 6-month dietary study with female heterozygous p53-deficient transgenic mice, phenolphthalein induced a significant increase in the incidence of malignant lymphoma of thymic origin (Dunnick et al., 1997).

The data available from epidemiology studies on laxative use are limited and not sufficient for evaluating the potential carcinogenicity from exposure to phenolphthalein.

Other Information Relating to Carcinogenesis or Possible Mechanisms of Carcinogenesis

The malignant thymic lymphomas induced by phenolphthalein in female heterozygous p53-deficient transgenic mice exhibited a loss of the normal p53 allele, suggesting the involvement of a mutagenic mechanism in tumor induction and/or progression (Dunnick et al., 1997).

Phenolphthalein causes enhanced oxygen radical production in *in vitro* systems. *In vivo*, reduction of phenoxyl radicals could allow reformation of phenolphthalein, establishing a futile cycle of oxidation and reduction, thereby generating more free radical species. Thus, phenolphthalein may be a significant source of oxidative stress in physiological systems.

Although negative for mutagenicity and DNA damage in bacteria, phenolphthalein exhibits genetic activity in several *in vitro* and *in vivo* mammalian assays. Phenolphthalein was positive for the induction of chromosomal aberrations in cultured Chinese hamster ovary cells in the presence of metabolic activation and induced *hprt* gene mutations, chromosomal aberrations, and morphological transformation in Syrian hamster embryo cells. Phenolphthalein was also positive for the induction of micronucleated erythrocytes in mice following multiple but not single treatments administered by gavage or dosed feed. Phenolphthalein also induced micronuclei in female heterozygous p53-deficient transgenic mice exposed via dosed feed for 26 weeks. Abnormal sperm were induced in male mice but not male rats treated with phenolphthalein via dosed feed for 13 weeks. Phenolphthalein was negative for Na/K ATPase gene mutations and aneuploidy in Syrian hamster embryo cells.

No data are available that would suggest that the mechanisms thought to account for tumor induction by phenolphthalein in experimental animals would not also operate in humans. Phenolphthalein causes oxidative stress and also demonstrates the capability to alter tumor suppressor gene pathways, which are both mechanisms believed to be involved in human cancer.

Listing Criteria from the Report on Carcinogens, Eighth Edition

Known To Be A Human Carcinogen:

There is sufficient evidence of carcinogenicity from studies in humans which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer.

Reasonably Anticipated To Be A Human Carcinogen:

There is limited evidence of carcinogenicity from studies in humans, which indicates that causal interpretation is credible, but that alternative explanations, such as chance, bias or confounding factors, could not adequately be excluded, or

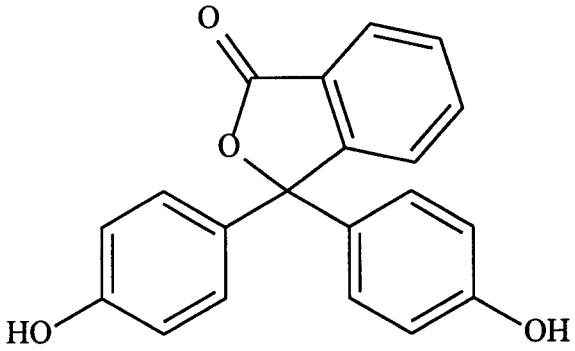
There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site or type of tumor, or age at onset; or

There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however; the agent, substance or mixture belongs to a well-defined, structurally related class of substances whose members are listed in a previous Report on Carcinogens as either a known to be human carcinogen or reasonably anticipated to be human carcinogen, or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.

1.0 CHEMICAL PROPERTIES

Phenolphthalein
[77-09-8]



1.1 Chemical Identification

Phenolphthalein ($C_{20}H_{14}O_4$, mol. wt. = 318.33) is also called:

1(3*H*)-Isobenzofuranone, 3,3-bis(4-hydroxyphenyl)- (9CI)
3,3-Bis(4-hydroxyphenyl)-1(3*H*)-isobenzofuranone
3,3-Bis(p-hydroxyphenyl)phthalide
3,3-Bis(4-hydroxyphenyl)phthalide
α-(*p*-Hydroxyphenyl)-α-(4-oxo-2,5-cyclohexadien-1-ylidine)-*o*-toluic acid
α-Di(*p*-hydroxyphenyl)phthalide
Dihydroxyphthalophenone
Phthalide, 3,3,-bis(*p*-hydroxyphenyl)
Phthalimetten®
Purgophen (VAN)
α,4,4′-Trihydroxytriphenylmethane-2-carboxylic acid lactone

Many commercial brand name laxatives have included phenolphthalein in their formulations. They include the following:

Agoral®
Alophen®
Chocolax®
Colax®
Correctol®
Darmol®
Dialose®Plus
Doxidan®
Espotabs®
Euchessina®
Evac-U-Gen®
Evac-Q-Kit®
Evac-Q-Kwik®
Evac-U-Lax®
Evac-Q-Tabs®
Ex-Lax®
Feen-A-Mint Gum®
FemiLax®

Kondremul®
Koprol®
Lactone
LaxCaps®
Laxogen®
Lax-Pills®
Lilo®
Medilax®
Modane®
Phenolax®
Phillips®
Phthlalin®
Prulet®
Purga®
Purgen®
Spulmako-lax®

In September 1997, the U.S. Food and Drug Administration (FDA) proposed reclassification of the use of phenolphthalein in over-the-counter (OTC) laxative products (FDA, 1997). In anticipation of the FDA action, the producers of Correctol® and Feen-a-Mint® brand products replaced phenolphthalein with bisacodyl in January 1996. Bayer's Phillips' GelCaps was voluntarily removed from the market in mid-1997; and Novartis AG, the marketer of Ex-Lax®, announced in late August 1997 that its product would be reformulated, substituting senna for phenolphthalein (Suplee, 1997; Drug Topics, 1997).

1.2 Physical-Chemical Properties

Property	Information	Reference
Color	White or yellow-white; pink to deep red in alkaline solution (pH>9); colorless to pH=8.5	Budavari (1996)
Physical State	Minute, triclinic crystals	Budavari (1996)
Melting Point, ° C	258-262	Budavari (1996)
Specific Gravity	1.299	Budavari (1996)
Dissociation Constant at 25°C (pKa)	9.7	Budavari (1996)
Odor	Odorless	NTP (1996)
Solubility:		
Water at 20°C	Almost insoluble in water.	Budavari (1996)
Organic Solvents	Soluble in alcohol and diethyl ether, very slightly soluble in chloroform. Soluble in dilute solutions of alkali hydroxides and hot solutions of alkali carbonates.	Budavari (1996)

2.0 HUMAN EXPOSURE

2.1 Use

Phenolphthalein in 1% alcoholic solution is used as a visual indicator in titrations of mineral and organic acids and most alkalies. Phenolphthalein-titrated solutions are colorless at pH < 8.5 and pink to deep-red at pH > 9. Phenolphthalein is not suitable, however, for titrating ammonia or alkaloids; carbonate solutions should be boiled before titration because of a high sensitivity to carbon dioxide (Budavari, 1996).

Phenolphthalein is used in a variety of ingested products and in some scientific applications. Because phenolphthalein is odorless and tasteless, it can be incorporated easily in tablets, powder, and liquid. It has been commonly used as a laxative, available worldwide as an over-the-counter chocolate or gum laxative product. The official dose is 60 mg, but adults usually require 100 to 200 mg (Fingl, 1965). Bedridden patients require 500-mg doses (Sollman, 1957). Dunnick and Hailey (1996) estimated that the low dose used in the 2-year NTP mouse bioassay is about 10 times a human dose of 5 mg/kg on a body surface area basis.

The use of laxatives to relieve constipation and to maintain regularity in bowel habits is widespread in the United States. Two large surveys of the U.S. adult population agreed on the prevalence of laxative use at least once per month (11.5% ca. 1989, 9.7% in 1982-1984) but differed in self-reported constipation (3.4% ca. 1989, 20.3% in 1982-1984). These studies and others agree that female users outnumber male users (e.g., 20.8% of females and 8.0% of males in 1971-1975 and 4.9% in females and 1.3% in males ca. 1989), that the fraction of users increases with age, and that persons who self-report constipation are more likely to use laxatives and stool softeners than persons who do not report constipation (e.g., 22.6% vs. 6.4% in 1982-1984) (Harari et al., 1989 [ca. 1989 data]; Everhart et al., 1989 [1971-1975 and 1982-1984 data]). Differences between the studies may lie in the relative ages of the subjects. In the ca. 1989 population, 47% was younger than 40, whereas in the 1982-1984 study, only 15% of the population was younger than 45.

Few studies report on the prevalence of phenolphthalein laxative use. To judge from the four populations described below, it would appear that no more than 10% of the U.S. population has used phenolphthalein-containing laxatives as often as once per month, but up to 5% may have used them weekly or more often.

One study of 424 cases of invasive adenocarcinoma of the colon and 414 controls in Washington state, ages 30 to 62 years, found that 13.6% of the subjects reported constipation requiring treatment (use of a laxative, enema, or prunes), 4.7% reported ever use of phenolphthalein laxatives, and 3.5% reported use of phenolphthalein laxatives at least 350 times in their lifetimes (Jacobs and White, 1998).

In three U.S. populations of 268 to 813 persons comprising approximately equal numbers of cases of adenomatous colorectal polyps and controls, 0.97 to 5.1% of the subjects used phenolphthalein laxatives at least once per week. The two North Carolina groups included subjects aged 30 to 89 years, 58% and 53% of which were female; the group in Los Angeles, California, included subjects aged 50 to 74 years of which 34% were female. Mean ages of the three groups were comparable (59 to 62 years). The frequent phenolphthalein laxative users represented 8 to 30% of all frequent laxative users. The ever use of phenolphthalein laxatives in the two North Carolina groups was 17.5% and 25%, with 10% and 7% using them at least once per month (Longnecker at al., 1997).

2.2. Producers, Production Process, and Production Volume

The only current U.S. producer of phenolphthalein is Sigma-Aldrich Corporation (SRI International, 1997a). In 1989, two of the U.S. producers of phenolphthalein were Aldrich Chemical Co, Inc., of Milwaukee, WI, and Hill Brothers Chemical Co., of Orange, CA (SRI International, 1989; cited by HSDB, 1997).

Phenolphthalein is produced commercially by condensation of phthalic anhydride with phenol in the presence of sulfuric acid, a dehydrating agent (Martin and Cook, 1961; Budavari, 1996).

The *Chemical Economics Handbook* (1992; cited by NTP, 1996) reported that 250 tons (197 metric tons or Mg) of phenolphthalein was produced annually by Sigma-Aldrich Corporation. Current production is reported to be the same (SRI International, 1997a).

Combined sales of the top 3 phenolphthalein-containing drugs, Correctol® by Schering Plough Health Care, Phillips® by the Bayer Corporation, and Ex-Lax® by Sandoz Pharmaceuticals (currently marketed by Novartis AG), totaled 16.4% of the laxative market in 1989 (Drug Store News, 1990); 23.9% in 1992 (Advertising Age, 1993); and 19.9% in the period July 1993-July 2, 1994 (DeNitto, 1994). The three drugs were still among the top-selling laxatives in 1995 (SRI International, 1997b)

Ex-Lax® was among the top 3 best-selling laxatives in 1996, accounting for about 7% of the brand-name sales. Phenolphthalein was removed from Correctol® in early 1996, and from Phillips GelCaps® and Ex-Lax® in 1997. In the latter 2 cases, the phenolphthalein-containing products were recalled from retail shelves (Suplee, 1997; Drug Topics, 1997).

U. S. FDA (1997) in its September 2 proposal to remove phenolphthalein from the generally recognized as safe and effective category estimated that the number of manufacturers of phenolphthalein-containing laxatives had fallen from the 60 manufacturers in its database to 20 manufacturers by August 1997.

2.3 Environmental Exposure

Exposure is possible through discharges to air and from process units where phenolphthalein is manufactured.

2.4 Occupational Exposure

A National Occupational Exposure Survey (NOES) conducted between 1981-1983 by the National Institute of Occupational Safety and Health (NIOSH) listed 75,243 workers (26% female) as being potentially exposed to phenolphthalein. The number of Health Services employees potentially exposed to phenolphthalein was 20,122 (65% female) (NIOSH, 1990; cited by NTP, 1996).

2.5 Regulations and Criteria

The U.S. Food and Drug Administration proposed on September 2, 1997, to declare all phenolphthalein-containing drug products to be new drugs within the meaning of Section 201(p) of the Federal Food, Drug, and Cosmetic Act [21 U.S.C. 321(p)]. In the *Federal Register* notice, which affects 21 CFR Part 310 and 334, FDA proposed to reclassify phenolphthalein from Category I (generally recognized as safe and effective and not misbranded) to Category II (not generally recognized as safe and effective and misbranded) and added it to a list of non-

monograph active ingredients. Phenolphthalein would be added to 21 CFR Section 310.545(a)(12)(iv), the list of stimulant laxatives (U.S. FDA, 1997).

The notice concluded with the statement: "the FDA considers use of phenolphthalein a potential risk to humans. These findings of rodent carcinogenicity and genotoxicity in several test systems indicate that chronic use could lead to damage to the human genome (including p53, which is known to be a tumor suppressor gene) and could increase the risk of malignancy." The FDA invited comments on these findings, and in late 1997 was reviewing written comments received from industry (U.S. FDA, 1997).

As described in Section 1.1, many over-the-counter phenolphthalein-containing laxatives were being reformulated in late 1997 without phenolphthalein.

REGULATIONS[a]

	Regulatory Action	Effect of Regulation/Other Comments
E P A	40 CFR 63—PART 63—NATIONAL EMISSION STANDARDS FOR HAZARDOUS AIR POLLUTANTS FOR SOURCE CATEGORIES. Promulgated: 57 FR 61992, 12/29/92. U.S. Code: 7401 et seq.	Standards that regulate specific categories of stationary sources that emit (or have potential to emit) one or more hazardous air pollutants (HAP) are listed in this part pursuant to section 112(b) of the CAA. Phenolphthalein itself is not an HAP.
	40 CFR 63.100 ff.—Subpart F—National Emission Standards for Organic Hazardous Air Pollutants From the Synthetic Organic Chemical Manufacturing Industry.	This subpart applies to chemical manufacturing process units that manufacture phenolphthalein (Group III) and are located at a plant site that is a major source as defined in section112(a) of CAA. Owners and operators of sources subject to this subpart shall comply with the requirements of subparts G and H of this part.
	40 CFR 63.110 ff.—Subpart G—National Emission Standards for Organic Hazardous Air Pollutants From the Synthetic Organic Chemical Manufacturing Industry for Process Vents.	The provisions of this subpart apply to all process vents, storage vessels, transfer racks, and wastewater streams within a source subject to subpart F of this part. Emission standard: Emissions of organic HAPs from phenolphthalein-manufacturing plants shall be controlled to the level represented by a given equation [see 40 CFR 63.112(a)]. Specific process vent and methods and procedures provisions apply.

REGULATIONS[a]

	Regulatory Action	Effect of Regulation/Other Comments
F D A	21 CFR 201—PART 201—LABELING. Promulgated: 40 FR 13998, 03/27/75. U.S. Code: 21 U.S.C.321, 331, 351, 352 355, 356, 357, 358, 360, 360b, 360gg-360ss, 371, 374, 379e; 42 U.S.C. 216, 241, 262, 264. 21 CFR 201.60 ff.—Subpart B—Labeling Requirements for Prescription Drugs and/or Insulin. 21 CFR 201.57—Sec. 210.57 Specific requirements on content and format of labeling for human prescription drugs.	On human drug prescriptions, there must be a subsection of the labeling stating whether long-term studies in animals have been performed to evaluate carcinogenic potential, and it must include the species and results. Any precautionary statements on these topics shall also include practical, relevant advice on the significance of these animal findings. If there is evidence from the human data that the drug may be carcinogenic or mutagenic or that it impairs fertility, this information will be contained in the "Warning" section.
	21 CFR 369—PART 369—INTERPRETATIVE STATEMENTS RE: WARNINGS ON DRUGS AND DEVICES FOR OVER-THE-COUNTER SALE. Promulgated: 39 FR 11745 03/29/74. U.S. Code: 21 U.S.C. 321, 331, 351, 352, 353, 355, 356, 357, 371.	Subparts A and B regulate the warning and caution statements included on the labels of drugs and devices for over-the-counter sale.
	21 CFR 369.20—Sec. 369.20 Drugs; recommended warning and caution statements.	Drug preparations containing phenolphthalein should contain, in addition to the general warning, the following statement: "*Caution*—If skin rash appears, do not use this or any other preparation containing phenolphthalein."
O S H A	29 CFR 1910—PART 1910—OCCUPATIONAL SAFETY AND HEALTH STANDARDS. Promulgated: 39 FR 23502, 06/27/74. U.S. Code: 29 U.S.C. 653, 655, and 657.	

REGULATIONS[a]

	Regulatory Action	Effect of Regulation/Other Comments
OSHA	29 CFR 1910.1200—Sec. 1910.1200 Promulgated 02/15/89. OSH. Act: Hazard Communication Standard.	Requires chemical manufacturers and importers and all employers to assess chemical hazards and to provide information to employees. Hazard Communication program to include labels, material safety data sheets, and worker training. Labels may be subject to FD & CA requirements.

[a] The regulations in this table have been updated through 62 Federal Register 23394, April 30, 1997.

3.0 HUMAN STUDIES

There are no adequate epidemiology studies specifically addressing the relationship between phenolphthalein use and human cancer. The National Cancer Institute nominated phenolphthalein for study because this was a widely used over-the-counter drug for which there were no adequate toxicity or carcinogenicity studies reported in the literature.

Human carcinogenicity studies reported prior to 1996 are reviewed in NTP (1996, pp. 19-24, see Appendix A). Studies not included in or published after NTP (1996) are summarized below and in **Table 3-1**.

No statistically significant increase in colorectal cancer risk was observed in 1,408 patients (selected from April 1980 to April 1981) of "The Melbourne Colorectal Cancer [Case-Control] Study" for those who used phenolphthalein laxatives (Kune, 1993; cited by NTP, 1996). Controls for this study were randomly selected from the same geographic area and age/sex frequency matched with the clinical cases. Also, in a case-control study of 11,888 California retirement community residents, the association between laxative use and the risk of colorectal cancer was not significant (Wu et al., 1987; cited by NTP, 1996).

Data from three case-control studies were examined to assess the relationship between the use of phenolphthalein-containing laxatives and the occurrence of adenomatous colorectal polyps (Longnecker et al., 1997). The case (730 total subjects) and control (907 total subjects) groups were part of three separate case-control studies conducted in Los Angeles (1991-1993) and North Carolina (1988-1990, 1992-1995). In all studies, subjects were selected from among those patients undergoing an endoscopic procedure. All cases had histologically confirmed adenomatous polyps. Controls in Los Angeles included subjects without polyps of any kind; in North Carolina patients had no adenomatous polyps. Data collected from subjects included frequency of use of phenolphthalein-containing laxatives and other dietary and nondietary factors. The overall prevalence of phenolphthalein-containing laxative use ranged from 1.3% to 4.2%.

The overall adjusted odds ratios for the two North Carolina studies (study-1 OR = 1.0, 0.4-2.2; study-2 OR = 1.1, 0.2 - 5.7) did not suggest that use of phenolphthalein-containing laxatives increased the risk of adenomatous colorectal polyps (Table 3-1), but the estimates had relatively wide confidence intervals because of the generally infrequent use of phenolphthalein containing laxatives. Alternatively, there was a slight association in the Los Angeles study (OR = 1.8, 0.5 - 6.2). However, no increase in risk was identified with increasing frequency of use in the L.A. group. The results were not altered by adjustment for a number of potentially confounding factors including constipation or bowel movement. There was no association in any study with the use of other types of laxatives. The study strengths include the use of data from multiple well-designed case-control studies and adjustment for a number of confounding factors. However, the small number of exposed subjects does not allow for a precise estimate of the relative risk associated with phenolphthalein-containing laxatives (Longnecker et al., 1997).

A retrospective cohort study of 2,277 German patients who underwent colonoscopy was conducted to examine laxative use and melanosis coli as a risk factor for colorectal neoplasms (Nusko et al., 1993). Data on laxative use was obtained from hospital records. A total of 271 patients (11.9% of all patients) reported using laxatives, among whom 15 developed colorectal cancer. Among those patients who did not report using laxatives (n = 2,006), 88 (4.4%) developed colorectal cancer. There was no statistically significant association between laxative use and risk of colorectal cancer [relative risk (RR) = 1.26, 95% confidence interval (CI) = 0.74-2.15, p = 0.485)]. However, there was a statistically significant association between the occurrence of colorectal adenomas and laxative use [RR = 1.72, 95% confidence interval (CI) = 1.46-2.01, p = 0.0001)]. Of the 164 patients (60%) for whom laxative ingredients were known, however, only two used phenolphthalein-containing products.

A recent meta-analysis of 14 case-control studies showed a small but significant association between colorectal cancer and use of cathartics (pooled odds ratio = 1.46 (1.33-1.61); Sonnenberg and Müller, 1993). Specific ingredients were not considered. The authors suggested that the risk may reflect the confounding influence of dietary habits rather than constipation or laxative use.

Table 3-1. Human Studies of Effects of Exposure to Phenolphthalein

Design	Population Group	Exposure	Effects	Potential Confounders/Effects	Comments	Reference
Case-controlled	**Cases:** 730 men and women with histologically confirmed adenomatous polyps; identified at three designated medical facilities: Los Angeles (L.A.), North Carolina 1 and 2 (NC1, NC2); individuals were excluded if evidence of previous bowel cancer, adenoma, bowel surgery, inflammatory bowel disease, polyposis, or could not speak English **Controls:** 907 men and women total; at L.A. had no polyps of any type and were individually matched to cases by age, sex, medical facility, and period of exam; at NC1 and NC2 had not adenomatous polyps and were not matched to cases	**Estimation:** Laxative use, dietary variables, and other nondietary variables collected by personal interview (L.A.) or by telephone (NC1 and NC2) **Duration:** L.A.: 1991-93; NC1: 1988-90; NC2: 1992-1995 **Response Rate (%):** L.A.-83; NC1-83; NC2-45 **Categories:** Laxative with/without phenolphthalein used at least once/week; laxatives classified as containing phenolphthalein if one of 19 brands reported	**Evaluation:** Calculation of Odds Ratio (OR) to assess relation between use of laxatives and risk of adenoma; logistic regression models used to adjust ratios for potentially confounding factors **OR (95% CI):** For use of phenolphthalein-containing laxatives once a week or more: L.A.: 1.3 (0.9-1.9) NC1: 1.0 (0.5-1.7) NC2: 0.9 (0.4-1.8)	OR adjusted a priori L.A.: alcohol, smoking, vigorous activity, intake of energy, saturated fat, fruits, and vegetables NC1 and NC2: age, sex, alcohol, smoking, intake of energy, total fat, fiber from fruits and vegetables NC1: also adjusted for 'leisure activity' NC2: also adjusted for 'hard physical activity' Additional adjustment for race, body mass index, use of nonsteroidal anti-inflammatory agents Adjustment of OR had no significant effect on the relation between laxative use and risk of adenoma	Prevalence of phenolphthalein-laxative use was less than 5%; the low prevalence probably accounted for wide confidence intervals	Longnecker et al. (1997)
Retrospective Cohort	2,277 patients (1,193 males and 1,084 females) diagnosed by colonoscopy. Results of colonoscopies classified as: normal, polyps, carcinomas, chronic inflammatory bowel disease, diverticulitis, operative anastomases (postresectional state), and melanosis coli	271 patients had a history of laxative use. Ingredients of laxatives used were known for 164 patients. Only two reported use of phenolphthalein-containing products	**Evaluation:** Calculation of Relative Risk (RR) to assess relation between use of laxatives and colorectal adenomas/carcinomas: **RR(95% CI)** of colorectal adenomas in patients using laxatives: 1.72 (1.43-2.01), p = 0.0001 **RR (95% CI)** of colorectal carcinomas in patients using laxatives: Not significant at p = 0.485	The authors noted that the following information was difficult to obtain: sufficient information about the intake of laxatives, and duration and frequency of laxative intake. Therefore, a causal relationship between laxative use and colorectal tumors was not clarified exactly in this study.	Number of individuals using phenolphthalein-containing laxatives was very low in this study (see exposure).	Nusko et al. (1993)

Table 3-1. Human Studies of Effects of Exposure to Phenolphthalein (Continued)

Design	Population Group	Exposure	Effects	Potential Confounders/Effects	Comments	Reference
Meta-analysis of 14 previously published case-control studies	11 of the 14 case-control studies contained information about laxative use prior to the onset of colorectal cancer. **Exposed:** 4,413 individuals reported use of cathartics **Controls:** 22,258 individuals reported no use of cathartics	In the 11 studies, cathartics were categorized under the following specifications of use: cascara, senna, Beecham's pills, salts, liquid paraffin (mineral oil), other cathartics, > weekly and constantly, laxatives >1/week for <10 years and more, suppositories and enemas 1-6/week, stool-bulk additives, ever used laxatives regularly, laxatives, chronic use of laxatives, laxatives >1/week, suppository and enemas 1-6/week, laxatives daily or weekly, self-reported commercial laxative use, and use of laxatives regularly/often	**Evaluation:** Pooled odds ratios of the colorectal cancer risk among exposed and unexposed subjects. **OR (95% CI)** for use of cathartics: 1.46 (1.33-1.61)	Multivariate analysis showed that low intake of fiber, vegetables, and vitamin C-containing foods and a high intake of fat have an important confounding effect.	The article did not provide ingredients in the cathartic agents. The percentage of these compounds containing phenolphthalein is unknown.	Sonnenberg and Müller (1993)

CI = confidence interval

4.0 EXPERIMENTAL CARCINOGENICITY

Dietary exposure to phenolphthalein for two years induced a significant increase in multiple types of tumors in male and female rats and mice (NTP, 1996, pp. 39-79, see Appendix A). The results of these studies are summarized below. More recently, dietary exposure to phenolphthalein for 6 months induced a significant increase in the incidence of malignant lymphoma of the thymus in female heterozygous p53-deficient transgenic mice (TSG-p53™) (Dunnick et al., 1997; see summary below and **Table 4-1**).

4.1 Rats

In the NTP two-year bioassay, male and female Fischer 344 rats were administered 12000, 25000, or 50000 ppm (37.7, 78.450, or 157.08 mmol/kg) phenolphthalein in the diet (NTP, 1996). Survival of all groups of exposed males and females was similar to that of controls.

The incidence of benign pheochromocytoma of the adrenal medulla was significantly increased in all dosed male groups. Most pheochromocytomas in dosed males were bilateral. Malignant pheochromocytoma was not affected. The incidence of benign pheochromocytoma was also increased in female rats exposed to 12000 ppm. Bilateral tumors and malignant pheochromocytoma were not affected in dosed females.

The incidence of renal tubule adenoma (single and step sections combined) was significantly increased in all dosed male groups. A few renal tubule carcinomas were also observed in dosed males. In females, one renal tubule adenoma was observed in the 50000-ppm dose group, but an extended evaluation failed to reveal additional tumors and this adenoma was not considered treatment-related.

4.2 Mice

In the NTP two-year bioassay, male and female $B6C3F_1$ mice were administered 3000, 6000, or 12000 ppm (9.4, 18.85, or 37.7 mmol/kg) phenolphthalein in the diet (NTP, 1996). Females treated with 12000 ppm had a significantly decreased rate of survival compared to controls.

The incidence of histiocytic sarcoma was significantly greater in 6000- and 12000-ppm males and females as compared to controls. The incidence of malignant lymphoma (all types) was significantly increased in exposed females, but not in males. The incidence of lymphoma of a thymic origin was significantly increased in exposed females and in 6000-ppm males. The incidence of benign ovarian sex-cord stromal tumors was significantly increased in exposed females. The incidence of hepatocellular adenoma or carcinoma was significantly reduced in exposed females and in 6000- and 12000-ppm males.

The heterozygous p53-deficient (+/-) mouse model has been proposed for rapid identification of carcinogenic responses of mutagenic chemicals (Tennant et al., 1995). To further characterize the mechanisms of phenolphthalein-induced carcinogenesis, a NIEHS study was conducted in the heterozygous p53-deficient (+/-) mouse (Dunnick et al., 1997). The p53 (+/-) mouse has a null mutation introduced into one p53 gene by homologous recombination in murine embryonic stem cells. The insertion of the *neo* cassette into the Trp53 locus resulted in a deletion of a 450-base-pair fragment containing 106 nucleotides of exon 5, and about 350 nucleotides of intron 4 that inactivated gene function (Donehower et al., 1992).

In the NIEHS study, p53 (+/-) female mice received phenolphthalein in the diet at 200, 375, 750, 3000, or 12000 ppm. The average daily dietary consumption of phenolphthalein was estimated as 43, 84, 174, 689, or 2375 mg/kg body weight/day [or 129, 252, 522, 2867 mg/m^2 body surface area/day (Freirich et al., 1966)]. Human exposure is approximately 5 mg/kg/day or 185 mg/m^2 body surface area/day under expected conditions of use (Dunnick and Hailey, 1996). The two lowest levels in the NIEHS transgenic study delivered phenolphthalein exposures that were approximately 0.5 to 1.5 times the recommended human exposure levels based on a mg/m^2 body surface area comparison.

In this study, the incidence of malignant lymphoma from the thymus was significantly increased in heterozygous p5-deficient female mice given 3000 or 12000 ppm (9.4 or 37.7 mmol/kg) phenolphthalein in the diet continuously for 26 weeks but not in mice given lower doses of 200, 375, or 750 ppm (0.6, 1.2, 2.4 mmol/kg diet). The incidence observed in the 0 to 12000-ppm dose groups was 0/19, 1/20, 1/20, 5/20, 20/20, and 19/20 (Dunnick et al., 1997).

Table 4-1. Experimental Carcinogenicity of Phenolphthalein Published Post NTP (1996)

Age, Strain, Species	No. and Sex Exposed	Controls	Chemical Form and Purity	Dose Route	Duration of Exposure	Results/Comments	Reference
Mice							
p53 transgenic mice	Five groups of 20 females	20 females	>99% pure article; administered diets ± 10% of scheduled dose	200, 375, 750, 3000, or 12000 ppm (mg/kg diet) (0.6, 1.2, 2.4, 9.4, or 37.7 mmol/kg diet) Estimated as 43, 84, 174, 689, or 2375 mg/kg bw and 129, 252, 522, 2867, or 7128 mg/m^2 body surface area per day	26 weeks	The incidence of malignant lymphoma of the thymus was increased at the 3000- and 12000-ppm dose levels (0/19, 1/20, 1/20, 5/20, 20/20, and 19/20). The 200 and 375-ppm dose levels delivered phenolphthalein exposures approximately 0.5 to 1.5 times the estimated human exposure levels (4 mg/kg/day) based on body surface area comparisons.	Dunnick at al. (1997)

5.0 GENOTOXICITY

Studies of the genotoxic effects of phenolphthalein have been reviewed and summarized by NTP (1996, p. 70, see Appendix A). Studies published after or not included in NTP (1996) are summarized below and in **Table 5-1**.

5.1 Genotoxicity Studies Summarized in NTP (1996)

Phenolphthalein was negative in the *Bacillus subtilis* rec assay for DNA damage in the absence of S9 metabolic activation. Similar results were reported for phenolphthalein's inability to induce *his* gene mutations in *Salmonella typhimurium*. A negative response was obtained in strains TA1535, TA1537, TA1538, TA98, and TA100 both with and without S9 activation using either the plate incorporation or preincubation methods.

In vivo, micronuclei were induced by phenolphthalein in male and female B6C3F1 mouse peripheral blood polychromatic (PCE) and normochromatic (NCE) erythrocytes via dosed feed for 13 weeks. Abnormal sperm and low sperm density were induced in male B6C3F1 mice but not F344 rats treated with phenolphthalein via dosed feed for 13 weeks.

5.2 Genotoxicity Studies Published Post NTP (1996)

Phenolphthalein induced a positive increase in *hprt* but not Na^+/K^+ ATPase gene mutations or aneuploidy in Syrian hamster embryo (SHE) cells exposed for 48 hours (Tsutsui et al., 1997).

A highly significant, reproducible increase in chromosomal aberrations was reported in phenolphthalein-exposed Chinese hamster ovary cells in the presence but not the absence of S9 activation (Witt et al., 1995). Phenolphthalein also induced a positive increase in chromosomal aberrations but not aneuploidy in SHE cells exposed for 6 (aberrations) or 48 (aneuploidy) hours (Tsutsui et al., 1997).

Similarly, a dose-dependent increase in morphological transformation was induced by phenolphthalein in SHE cells exposed for 48 hours (Tsutsui et al., 1997).

In vivo, phenolphthalein induced an equivocal increase in DNA single-strand breaks in the peripheral blood leukocytes of female p53-deficient transgenic mice (C57Bl/6 background) following dosed-feed exposure for 26 weeks and sampling at days 39, 92, 137, and 183 (Tice et al., 1998). No increase was observed in the livers of treated mice sampled upon termination of exposure.

Micronuclei were induced by phenolphthalein in male and female B6C3F1 mouse peripheral blood polychromatic (PCE) and normochromatic (NCE) erythrocytes via a variety of exposures including gavage for 2 to 3 days, or dosed feed for 4 to 14 days (Witt et al., 1995). Similarly, phenolphthalein induced micronuclei in the blood PCE of CD-1 mice after 14 weeks of continuous feed treatment (Witt et al., 1995). Micronuclei were also induced in the bone marrow of male B6C3F1 mice treated by gavage for 3 days or feed for 4 to 14 days (Witt et al., 1995). No significant increases in micronuclei were observed in mouse bone marrow after only 2 days of gavage treatment or 3 days of feed treatment (Witt et al., 1995). A positive response was also observed in the peripheral blood PCE and NCE of female p53-deficient transgenic mice treated via dosed feed for 26 weeks and sampled at days 39, 92, 137, and 183 (Tice et al., 1998). Kinetochore analysis at the top dose showed that micronuclei were induced primarily from whole chromosomes rather than from breaks resulting in chromosomal fragments. The

lowest effective dose (LED) for the induction of micronucleated erythrocytes in female heterozygous p53-deficient mice by phenolphthalein was 200 ppm.. Based on surface area, the amount of phenolphthalein ingested at this dose (i.e., ~30 mg/kg/day = ~90 mg/m^2 body surface) is within the dose range anticipated for human consumption (~5 mg/kg/day or ~185 mg/m^2 body surface area) (Dunnick and Hailey, 1996).

Table 5-1. Summary of Phenolphthalein Genotoxicity Studies Published Post NTP (1996)

System	Biological Endpoint	S9/Other Metabolic Activation	Form and Purity	Doses Used	Endpoint Response -/+ Activation	Comments	Reference
5.2 Mammalian Systems *in vitro*							
Syrian hamster embryo (SHE) cells	*hprt* and Na^+/K^+ ATPase gene mutations	-	>98%	10, 20, and 40 µM for 48 h	positive (*hprt*), negative (ATPase)	Treatment induced a dose-dependent increase in mutant frequency (LED = 20 µM) at the *hprt* locus only.	Tsutsui et al. (1997)
Chinese hamster ovary (CHO) cells	chromosomal aberrations	-/+	NG in source used	11, 23, 30, 40, and 50 µg/mL for 16 h -S9 and 62 +S9	negative/ positive	Highly significant increases observed in two trials with S9 activation (LED = 40 µg/mL).	Witt et al.. (1995)
Syrian hamster embryo (SHE) cells	chromosome aberrations and aneuploidy	-	>98%	10, 20, and 40 µM for 6 h (aberrations) or 48 h (aneuploidy)	positive (aberrations), negative (aneuploidy)	A statistically significant level of chromosomal aberrations was induced at the highest dose only (40 µM) with no corresponding aneuploidy at any dose.	Tsutsui et al. (1997)
Syrian hamster embryo (SHE) cells	morphological transformation	-	>98%	10, 20, and 40 µM for 48 h	positive	Treatment induced a dose-dependent increase in percent transformation (LED = 10 µM).	Tsutsui et al. (1997)
5.2 Mammalian Systems *in vivo*							
Female heterozygous p53-deficient transgenic mice (C57B1/6 background) 20/dose	DNA single-strand breaks in peripheral blood leukocytes and liver cells (single cell gel assay)	n.a.	>99%	200, 375, 750, 3000, and 12,000 ppm in feed, continuously for 26 wk	equivocal (blood), negative (liver)	Blood smears were prepared on days 39, 92, 137, and 183 of treatment; liver, at termination. Significant increase in DNA damage was observed in blood leukocytes on day 39 and 137 but not days 92 and 183, making results inconclusive.	Tice et al. (1998)

Table 5-1. Summary of Phenolphthalein Genotoxicity Studies Published Post NTP (1996) (Continued)

System	Biological Endpoint	S9/Other Metabolic Activation	Form and Purity	Doses Used	Endpoint Response -/+ Activation	Comments	Reference
male B6C3F$_1$ mice	micronuclei induction in bone marrow and peripheral blood erythrocytes	n.a.	NG in source used	1000-4000 mg/kg/d gavage for 2-3 d (single or multiple injections per day); 4000 and 6000 mg/kg/d feed for 3-14 d	positive in both tissues	Positive response in blood and bone marrow PCE (LED = 6000 mg/kg). Negative response was observed in 2-day single injection gavage and 3-day dosed feed treatments.	Witt et al. (1995)
male CD-1 mice	micronuclei induction in peripheral blood erythrocytes	n.a.	NG in source used	120-3500 mg/kg/d via feed for 14 wk	positive	Positive response in peripheral blood PCE (LED = 120 mg/kg/day).	Witt et al.. (1995)
female heterozygous p53-deficient transgenic mice (C57B1/6 background) 20/dose	micronuclei induction in peripheral blood erythrocytes	n.a.	>99%	200, 375, 750, 3000, and 12,000 ppm in feed, continuously for 26 wk	positive	Blood smears were prepared on days 39, 92, 137, and 183 of treatment. At each sample time a highly significant level of micronucleated NCE (LED = 200 ppm) and PCE (LED = 375 ppm) was observed. Micronuclei were induced predominantly from numerical chromosomal damage (kinetochore-positive micronuclei were present at the top dose tested).	Tice et al. (1998)

HID = highest ineffective dose; LED = lowest effective dose; n.a. = not applicable; NG = not given

6.0 OTHER RELEVANT STUDIES

6.1 Absorption, Distribution, Metabolism, and Excretion

6.1.1 Absorption and Metabolism

Phenolphthalein is absorbed in the intestine (Visek et al., 1956; AHFS, 1995; cited by NTP, 1996) and is almost completely converted to its glucuronide during extensive first pass metabolism in the intestinal epithelium and liver (Parker et al., 1980; cited by NTP, 1996) via uridine diphosphate glucuronosyltransferase (UDPGT) (Sund and Hillestad, 1982; cited by NTP, 1996). In the guinea pig, small amounts of sulfate conjugate metabolites have been detected in isolated mucosal sheets originating in the jejunum and colon (Sund and Lauterbach, 1986; cited by NTP, 1996).

Six hours after female Wistar rats were given [^{3}H]phenolphthalein, analysis of the systemic circulation showed that all of the radioactivity was associated with the glucuronide conjugate (Colburn et al., 1979; cited by NTP, 1996). Five to six hours after i.v. administration, absorption of [^{3}H]phenolphthalein from the intestine coincided with a secondary peak in blood radioactivity that followed hydrolysis by bacterial β-glucuronidase of [^{3}H]phenolphthalein glucuronide excreted in the bile. Enterohepatic recirculation is rate-limited by the hydrolysis of phenolphthalein glucuronide to aglycone (Bergan et al., 1982; cited by NTP, 1996).

The extent of enterohepatic recirculation of phenolphthalein was examined in rats after surgical cannulation of the bile duct (Parker et al., 1980; cited by NTP, 1996). Within 24 hours, 95% of 25 mg [^{3}H]phenolphthalein/kg administered intraperitoneally (i.p.) to female Wistar rats was recovered as glucuronide in the bile, with 0.2% recovery from the urine. In rats that were not surgically altered, 86% of the same dose was recovered in the feces, with little glucuronide, and 10% was recovered in the urine, primarily as the glucuronide. In female Wistar rats with biliary fistulae, 100% of the dose was eliminated in the bile, with 98% in the glucuronide form (Millburn et al., 1967; cited by NTP, 1996).

In studies of male Sprague-Dawley CR-1 strain rats whose femoral vein, artery, and bile duct were cannulated, i.v. administration of 3, 30, or 60 mg (9, 94, or 190 μmol) phenolphthalein led to elimination of 99.5% of the dose in the bile as the glucuronide. When the same rats were given 3, 30, or 100 mg (6, 59, or 195 μmol) phenolphthalein glucuronide by i.v. administration, no phenolphthalein was detected in the bile (Mehendale, 1990; cited by NTP, 1996).

Within 72 hours of oral administration of 4.8 mg [^{14}C]phenolphthalein/kg to female dogs (unspecified breed), 51% of the radioactivity was excreted in the feces and 36% was eliminated in the urine. After an i.v. dose, 54% in feces and 37% in urine were found. When the same dogs were bile-duct cannulated, an oral dose led to 31% radioactivity in feces, 38% in urine, and 22% in bile. An i.v. dose resulted in 11% elimination in feces, 35% in urine, and 43% in bile (Visek et al., 1956; cited by NTP, 1996).

6.1.2 Distribution

Studies done in dogs and mice using [^{14}C]phenolphthalein showed the radioactivity at levels parallel to the concentration in the blood in a wide and even distribution. Other experiments showed less than 0.03% of the dose in the liver and gall bladder and no radiolabel in the blood of newborn puppies, following administration of a 4.8 mg/kg (0.015 mmol/kg) oral

dose to the mother 50 hours prior to her giving birth. This result led investigators to suggest extremely limited passage across the placenta (Visek et al., 1956; cited by NTP, 1996).

6.1.3 Excretion

Bile, urine, feces, and milk are all routes of excretion for phenolphthalein (Visek et al., 1956; AHFS, 1995; both cited by NTP, 1996). Seventy-two hours after administration of a radioactive oral dose to dogs, 50% was recovered in the feces and 36% in the urine. Studies of mice reported 56% of an oral dose recovered from the urine within 48 hours and an additional 38% recovered from the feces. When an i.v. dose was given, 30% was recovered from the urine and 68% from the feces (Visek et al., 1956; cited by NTP, 1996). Some phenolphthalein is excreted into the bile, and the prolonged cathartic effect may be due to the ensuing enterohepatic recirculation (Gilman et al., 1980; cited by HSDB, 1997). Pre-treatment with hepatic-microsomal-enzyme inducers increased biliary excretion of metabolites in rats, but a dose of phenolphthalein followed by enzyme inhibitors decreased it. Dosing with metabolites yielded no effects (Chemical Society, 1972; cited by HSDB, 1997).

6.2 Pharmacokinetics

The systemic blood concentration-time profile of phenolphthalein for the 24 hours following single i.v. bolus injection was described by a classical compartmental pharmacokinetics model, which indicated a long half-life (Colburn et al., 1979; cited by HSDB, 1997). There are indications that this long half-life is an artifact of enterohepatic recirculation.

Whole-body autoradiography studies of male BOM:NMRI mice showed high levels of radioactivity in the stomach, gall bladder, and small intestine 10 to 20 minutes after administration of an intragastric dose of 1 mL/kg [^{14}C]phenolphthalein (10 μCi/100g) (Sund et al., 1986; cited by NTP, 1996). As evidenced by the presence of radioactivity in peripheral organs (including the kidney, liver, and skin), the compound was absorbed from the gastrointestinal tract and moved through the intestinal tract. After 2 hours, it arrived at the large intestine; 4 hours after administration, maximum radioactivity was observed in the rectum. Two days after administration, no radiolabel was detected.

6.3 Structure-Activity Relationships

6.3.1 Diphenylmethane Structural Analogs

Phenolphthalein and several structurally related chemicals (e.g. bisacodyl.) have been used as laxatives (Sund, 1983).

Phenolphthalein is a derivative of diphenylmethane (Binder, 1977; cited by NTP, 1996). Other compounds containing the diphenylmethane substructure include bisacodyl [603-50-9], picosulfate [10040-45-6], oxyphenisatin [125-13-3] and its salts (oxyphenisitin diacetate [115-33-3], oxyphenisatin disulfide [37811-54-4]), *p,p'*-diaminodiphenylmethane (DDPM) [101-77-9], and phenol red [143-74-8]. Bisacodyl (used in laxatives) induces both calculi and epithelial proliferative lesions (including transitional cell carcinoma) in the urinary bladder of rats (Toyoda et al., 1993, 1994). Geboes et al. (1993) stated that bisacodyl and picosulfate (used in laxatives) have no major influence in colonic and ileal epithelial cell proliferation and should not be regarded as a tumor-promoting substances. Yang et al. (1993) also found in one study that bisacodyl did not promote early precancerous lesions in colonic epithelial cells of rats.

Oxyphenisatin and its salts (used in laxatives) are known to induce liver damage (Dietrichson et al., 1976; Delchier et al., 1979; Sund, 1983; and Homberg et al., 1985).

Phenol red (a widely used pH indicator) has shown growth factor and estrogen effects in cancer cell lines (Devleeschouwer et al., 1992; Welshons et al., 1988; Glover et al., 1988; Bindal and Katzenellenbogen, 1988). In contrast, another study found that phenol red had no effect on the growth of normal human, mouse, and rat mammary cells (Richards et al., 1988).

The compound *p, p'*-diaminodiphenylmethane (DDPM) (used as an epoxy resin hardener) was found to be carcinogenic (Hirose et al., 1986), but since it is an aromatic amine and carcinogenic effects would be expected, it is not considered further here.

6.3.2 Alkylating Substructure

The probability that opening of the 5-atom lactone ring in phenolphthalein would produce an alkylating carbocation was noted by Benigni et al. (1996) and Huff et al. (1996).

6.3.3 Physical-Chemical Properties

The log P (log of the octanol-water partition coefficient) of phenolphthalein is 0.95, indicating it is a hydrophobic compound that is retained in tissues. Additionally K_e (an electrophilicity parameter) has been calculated for phenolphthalein as 3.597, which is considered high. High K_e values seem to indicate a directly acting carcinogen's general chemical reactivity, its tendency to undergo a reductive metabolism, or its ability to attack DNA (Benigni et al., 1996).

7.0 MECHANISMS OF CARCINOGENESIS

7.1 Ovarian Cancer

Phenolphthalein exposure caused an increased incidence of ovarian neoplasms in mice as discussed in section 4 (NTP, 1996). These neoplasms were of sex-cord stromal origin and did not follow the normal path of ovarian tumorigenesis, which generally starts with the destruction and/or loss of oocytes and ends in gonadotropin-stimulated tumor growth (NTP, 1996; Dunnick and Hailey, 1996). Instead, phenolphthalein might stimulate cellular proliferation in the ovary in part through its competitive binding to the estrogen receptor (NTP, 1996; Ravdin et al., 1987; cited by Dunnick and Hailey, 1996). Estradiol can stimulate proliferation in ovarian cells (Rao et al., 1978; cited by NTP, 1996) and it is possible that phenolphthalein could mimic this behavior.

7.2 Radical Formation

If phenolphthalein is converted to a quinoid, it can induce free radical formation (NTP, 1996). Two phenolic substituents are present in phenolphthalein and neither has substituents *ortho* to the hydroxyl group. Thus, phenolphthalein should be readily oxidized to phenoxyl free radicals. In electron paramagnetic resonance (EPR) studies done on horseradish peroxidase (HRP) oxidation of the compound, a primary phenolphthalein phenoxyl radical was observed in addition to a larger signal that was likely due to polymeric free radical species. In addition to detection of the phenoxyl radicals, indirect results of the presence of a phenoxyl radical such as production of superoxide, glutathione thiyl, and ascorbate radicals, have been detected by EPR (Sipe et al., 1997). Further evidence for the existence of phenoxyl radicals comes from the observation that phenolphthalein stimulates intestinal prostaglandin formation (Beubler and Juan,

1978a, 1978b; Capasso et al., 1985, 1988; all cited by Sipe et al., 1997). Markey et al. (1987; cited by Sipe et al., 1997) found that the ability of compounds to act as peroxide substrates correlates directly with their ability to stimulate prostaglandin formation. Thus the prostaglandin-stimulating action of phenolphthalein is indirect evidence that it is metabolized to a phenoxyl free radical (Sipe et al., 1997).

As described in section 6.2, phenolphthalein is rapidly metabolized to its glucuronide. In the studies of Sipe et al. (1997), the same qualitative oxidation reactions seen with phenolphthalein were seen with its glucuronide. While the glucuronide was substantially less active than its parent compound, its high concentration in the body could mean that it is the primary source of phenoxyl radicals *in vivo* (Sipe et al., 1997).

Reduction of phenoxyl radicals *in vivo* can be accomplished by reduced glutathione , NADH, or ascorbate and could allow reformation of phenolphthalein, establishing a cycle of oxidation and reduction. This futile metabolism of both phenolphthalein and phenolphthalein glucuronide can stimulate oxidation of ascorbate, glutathione, and NAD(P)H followed by reaction of the newly produced free radicals with oxygen, resulting in production of superoxide (Sipe et al., 1997). Superoxide is converted to hydrogen peroxide and molecular oxygen in the presence of superoxide dismutase (SOD). Hydrogen peroxide can be a source of hydroxyl radical in the presence of reduced iron, and the highly reactive hydroxyl radical has been found to react with DNA, yielding a variety of products (Mouret et al., 1991; cited by Sipe et al., 1997).

7.3 Genetic Toxicity

Results of investigations of the mutagenicity of phenolphthalein provide evidence of its genotoxicity. For example, studies on mice exposed to phenolphthalein have shown significant increases in micronucleated PCEs and NCEs (Tice et al., 1998; Dietz et al., 1992). Doses greater than or equal to 2,000 mg/kg per day for at least two days induced micronuclei in erythrocytes, which could be detected in either bone marrow or blood. Doses of 120 mg/day when given over a longer period of time (14 weeks) were also highly effective in inducing micronucleated erythrocytes (Witt et al., 1995).

Tice et al. (1998) found that in female transgenic heterozygous p53-deficient mice evaluated under chronic exposure conditions, the induced micronuclei contained predominantly whole chromosomes. The lowest effective dose inducing a genotoxic response, micronucleated NCE, was 200 ppm. In this *in vivo* test system, phenolphthalein caused genetic damage at doses comparable to human therapeutic doses on a mg/m^2 basis. U.S. FDA (1997) stated that low doses were about 15 times the human exposure.

The malignant thymic lymphomas induced by phenolphthalein in female heterozygous p53-deficient transgenic mice exhibited a loss of the normal p53 allele, suggesting the involvement of a mutagenic mechanism in tumor induction and/or progression (Tice et al., 1998).

7.4 Thymic Lymphoma and Estrogenic Effects

NTP has studied approximately 400 chemicals in long-term bioassays in F344 rats and $B6C3F_1$ mice. Only three chemicals have been found to cause thymic lymphomas in mice, including phenolphthalein, butadiene, and ddC (2′,3′-dideoxycytidine). There is no indication that any of the estrogenic chemicals studied caused thymic lymphomas, e.g. DDT (NCI, 1978) or zearalenone (NTP, 1982). Phenolphthalein also caused thymic lymphomas in the p53-deficient

mouse, and in all tumors examined there was loss of the wild p53 allele. The wild p53 allele remains in many of the spontaneous tumors in the p53-deficient mice (Harvey et al., 1993; cited by Dunnick et al., 1997). These studies show that the mechanism for tumor formation in the phenolphthalein-induced thymic lymphomas in the p53-deficient mice probably involves the loss of p53 function (Dunnick et al., 1997).

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APPENDIX A

Excerpt from the NTP Technical Report
Toxicology and Carcinogenesis Studies of Phenolphthalein
in F344/N Rats and B6C3F$_1$ Mice (Feed Studies),
pp. 4-38, 71-91, November 1996

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS STUDIES OF PHENOLPHTHALEIN

(CAS NO. 77-09-8)

IN F344/N RATS AND B6C3F$_1$ MICE

(FEED STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

November 1996

NTP TR 465

NIH Publication No. 97-3390

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

CONTENTS

ABSTRACT 5

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY 12

TECHNICAL REPORTS REVIEW SUBCOMMITTEE 13

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS 14

INTRODUCTION 17

MATERIALS AND METHODS 27

RESULTS 39

DISCUSSION AND CONCLUSIONS 71

REFERENCES 81

APPENDIX A Summary of Lesions in Male Rats in the 2-Year Feed Study of Phenolphthalein 93

APPENDIX B Summary of Lesions in Female Rats in the 2-Year Feed Study of Phenolphthalein . . . 137

APPENDIX C Summary of Lesions in Male Mice in the 2-Year Feed Study of Phenolphthalein 175

APPENDIX D Summary of Lesions in Female Mice in the 2-Year Feed Study of Phenolphthalein . . 219

APPENDIX E Genetic Toxicology 259

APPENDIX F Organ Weights and Organ-Weight-to-Body-Weight Ratios 269

APPENDIX G Hematology and Clinical Chemistry Results 275

APPENDIX H Determinations of Total Phenolphthalein in Plasma 281

APPENDIX I Reproductive Tissue Evaluations and Estrous Cycle Characterization 291

APPENDIX J Chemical Characterization and Dose Formulation Studies 295

APPENDIX K Feed and Compound Consumption in the 2-Year Feed Studies of Phenolphthalein . . . 309

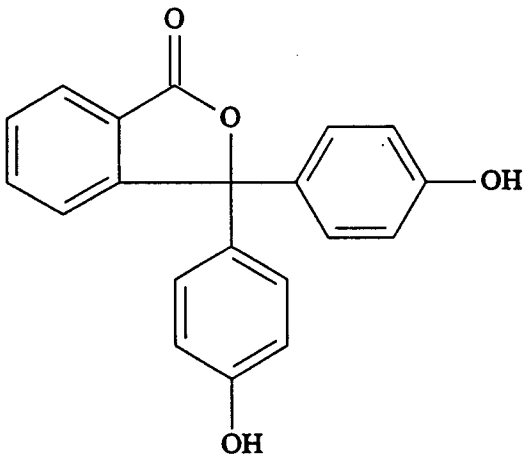
APPENDIX L Ingredients, Nutrient Composition, and Contaminant Levels in NIH-07 Rat and Mouse Ration 315

APPENDIX M Sentinel Animal Program 319

APPENDIX N Continuous Breeding Study in Swiss (CD-1®) Mice 323

APPENDIX O Single-Dose Toxicokinetic Studies in F344/N Rats and B6C3F$_1$ Mice 339

ABSTRACT



PHENOLPHTHALEIN

CAS No. 77-09-8

Chemical Formula: $C_{20}H_{14}O_4$ Molecular Weight: 318.33

Synonyms: 3,3-Bis(4-hydroxyphenyl)-1(3H)-isobenzofuranone; 3,3-bis(*p*-hydroxyphenyl)phthalide; α-(*p*-hydroxyphenyl)-α-(4-oxo-2,5-cyclohexadien-1-ylidene)-*o*-toluic acid

Trade names: Agoral®, Alophen®, Colax®, Correctol®, Dialose®, Doxidan®, Espotabs®, Evac-U-Gen®, Evac-U-Lax®, Ex-Lax®, Feen-A-Mint®, FemiLax®, Kondremul®, LaxCaps®, Lax-Pills®, Medilax®, Modane®, Phenolax®, Prulet®

Phenolphthalein is used as a laboratory reagent and acid-base indicator and in over-the-counter laxative preparations. The National Cancer Institute nominated phenolphthalein for study because of its widespread use as a component in numerous laxative preparations and the lack of adequate testing for carcinogenicity in experimental animals. Male and female F344/N rats and B6C3F$_1$ mice were exposed to phenolphthalein (98% to 99% pure) in feed for 14 days, 13 weeks, or 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium*, cultured Chinese hamster ovary cells, and mouse peripheral blood.

14-Day Study in Rats

Groups of five male and five female F344/N rats were given 0, 6,250, 12,500, 25,000, 50,000, or 100,000 ppm phenolphthalein in feed for 14 days. All rats survived to the end of the study. The final mean body weights of all exposed groups of rats were similar to those of the controls. No chemical-related gross or microscopic lesions were observed.

14-Day Study in Mice

Groups of five male and five female B6C3F$_1$ mice were given 0, 6,250, 12,500, 25,000, 50,000, or

100,000 ppm phenolphthalein in feed for 14 days. All mice survived to the end of the study. The final mean body weights of all exposed groups of mice were similar to those of the controls. No chemical-related gross or microscopic lesions were observed.

13-Week Study in Rats

Groups of 10 male and 9 or 10 female F344/N rats were given 0, 3,000, 6,000, 12,000, 25,000, or 50,000 ppm phenolphthalein (equivalent to average daily doses of approximately 200, 400, 800, 1,600, or 3,500 mg phenolphthalein/kg body weight to males and 200, 400, 800, 1,700, or 3,600 mg/kg to females) in feed for 13 weeks. Additional groups of 10 male and 10 female rats designated for clinical pathology evaluations were also given 0, 3,000, 6,000, 12,000, 25,000, or 50,000 ppm phenolphthalein in feed until day 21. All core study rats survived to the end of the study. The final mean body weight of the 50,000 ppm females and the mean body weight gains of the 25,000 and 50,000 ppm females were significantly lower than those of the controls. The final mean body weights and mean body weight gains of all other exposed groups were similar to those of the controls. There was no cathartic action or any other clinical finding attributed to exposure to phenolphthalein. The few differences in the hematology and clinical chemistry parameters were sporadic and were not considered to be chemical related. The percentage of motile sperm in the 12,000 ppm males was significantly greater than that in the controls, but no other significant differences in sperm morphology or vaginal cytology between exposed and control groups were observed. Absolute and relative liver weights of 25,000 and 50,000 ppm males were significantly greater than those of the controls. No chemical-related gross or microscopic lesions were observed.

13-Week Study in Mice

Groups of 10 male and 10 female B6C3F$_1$ mice were given 0, 3,000, 6,000, 12,000, 25,000, or 50,000 ppm phenolphthalein (equivalent to average daily doses of approximately 500, 1,000, 2,000, 4,100, or 9,000 mg phenolphthalein/kg body weight to males and 600, 1,200, 2,400, 5,000, or 10,500 mg/kg to females) in feed for 13 weeks. All mice survived until the end of the study. The final mean body weights and mean body weight gains of all exposed groups were similar to those of the controls. There was no cathartic action or any other clinical finding attributed to exposure to phenolphthalein. The absolute right cauda weight of the 12,000 ppm males and the absolute right epididymis weights of 12,000, 25,000, and 50,000 ppm males were significantly less than those of the controls. The percentages of abnormal sperm in 12,000, 25,000, and 50,000 ppm males were significantly greater than that in the control group, and the sperm concentrations in 12,000 and 50,000 ppm males were significantly less than that of the control group. The absolute and relative right testis weights of males exposed to 6,000 ppm or greater and the absolute right testis weight of 3,000 ppm males were significantly less than those of the controls. The incidences of hypoplasia of the bone marrow in males and females exposed to 12,000 ppm or greater were significantly greater than those in the controls. The incidences of hematopoiesis of the spleen in 25,000 and 50,000 ppm males were significantly greater than that in the controls.

2-Year Study in Rats

Groups of 50 male and 50 female F344/N rats were given 0, 12,000, 25,000, or 50,000 ppm phenolphthalein (equivalent to average daily doses of approximately 500, 1,000, or 2,000 mg phenolphthalein/kg body weight to males and 500, 1,000, or 2,500 mg/kg to females) in feed for 2 years.

Survival, Body Weights, and Clinical Findings

Survival of exposed males and females was similar to that of the controls. The mean body weights of exposed males were less than those of the controls through most of the second year of the study, and the mean body weights of exposed females were less than those of the controls from about week 16 until the end of the study. Clinical findings attributed to phenolphthalein exposure included thin appearance and ruffled fur in all exposed groups of males.

Determinations of Total Phenolphthalein in Plasma

The mean plasma concentrations of total phenolphthalein (free and conjugated) after 2 years of exposure varied little with time of day. Plasma concentrations of total phenolphthalein were approximately the same between exposure groups and between males and females.

Pathology Findings

The incidences of benign pheochromocytoma of the adrenal medulla in all exposed groups of males were significantly greater than those in the controls and occurred with a significant positive trend. The incidences of benign pheochromocytoma in 12,000 ppm females and of benign or malignant pheochromocytoma (combined) in 12,000 and 25,000 ppm females were significantly greater than those in the controls. The numbers of exposed males with bilateral benign pheochromocytomas exceeded the number of controls with these neoplasms. The incidences of malignant pheochromocytomas in exposed rats were similar to those in the controls. The incidences of focal hyperplasia of the adrenal medulla in the 12,000 and 50,000 ppm males were significantly greater than in the controls.

The incidences of renal tubule adenoma in 50,000 ppm male rats and of renal tubule adenoma or carcinoma (combined) in 12,000 and 50,000 ppm male rats were significantly greater than those in the controls. Although the increased incidences were predominantly of renal tubule adenoma, four carcinomas were observed in exposed males (0 ppm, 0/50; 12,000 ppm, 1/50; 25,000 ppm, 1/50; 50,000 ppm, 2/50). The incidences of renal tubule neoplasms in exposed groups of females were similar to those in the controls. The findings from an extended evaluation (step section) of the kidneys of female rats were similar to those from the standard evaluation. The incidences of nephropathy in all exposed groups of females were significantly greater than in the controls, and the severity of nephropathy in all exposed groups of males and in 25,000 and 50,000 ppm females was significantly greater than in the controls.

The incidences of diffuse hyperplasia of the parathyroid gland (0/41, 16/48, 14/49, 14/46), fibrous osteodystrophy of the bone (0/50, 17/50, 14/50, 12/50), and mineralization (0/50, 11/50, 5/50, 5/49) and degeneration (0/50, 11/50, 5/50, 4/49) of the glandular stomach in exposed groups of males were generally significantly greater than those in the controls. The incidences of hyperplasia of the thyroid gland C-cells (13/50, 3/50, 9/49, 4/49) in 12,000 and 50,000 ppm males were significantly less than in the controls. These lesions are commonly observed in male rats with more advanced nephropathy and are considered to be associated with a calcium/phosphorus imbalance created by compromised functional capacity of the kidney.

2-YEAR STUDY IN MICE

Groups of 50 male and 50 female B6C3F$_1$ mice were given 0, 3,000, 6,000, or 12,000 ppm phenolphthalein (equivalent to average daily doses of approximately 300, 600, or 1,200 mg phenolphthalein/kg body weight to males and 400, 800, or 1,500 mg/kg to females) in feed for 2 years.

Survival, Body Weights, and Clinical Findings

Survival of the 12,000 ppm females was significantly lower than that of the controls; survival of all other exposed groups of mice was similar to that of the controls. The mean body weights of 12,000 ppm males were slightly less than those of the controls beginning at week 93 of the study, and the mean body weights of the 3,000, 6,000, and 12,000 ppm females were less than those of the controls during most of the second year of the study. In exposed mice, there were no clinical findings related to phenolphthalein exposure.

Determinations of Total Phenolphthalein in Plasma

The mean plasma concentrations of total phenolphthalein (free and conjugated) after 2 years of exposure varied little with time of day. Plasma concentrations of total phenolphthalein were approximately the same between exposure groups and between males and females.

Pathology Findings

The incidences of histiocytic sarcoma in 6,000 and 12,000 ppm males and females were significantly greater than those in the controls and occurred with a significant positive trend. In this study, histiocytic sarcoma was consistently observed in the liver with several other sites (e.g., spleen, lung, bone marrow, and various lymph nodes) involved less frequently.

The incidences of all types of malignant lymphoma and of lymphoma of thymic origin in all exposed groups of females were significantly greater than those in the controls and occurred with significant positive trends, while the incidences of all types of malignant lymphoma in all exposed groups of males were similar to that in the controls. The incidences of lymphoma of thymic origin were increased in exposed groups of males, but were significantly increased only in the 6,000 ppm group. The incidences of atypical hyperplasia of the thymus in 6,000 and 12,000 ppm males and in all exposed groups of females were significantly greater than those in the controls.

The incidences of benign sex-cord stromal tumors of the ovary in all exposed groups of females were significantly greater than in the controls. The incidences of hyperplasia of the ovary in 3,000 and 12,000 ppm females were significantly greater than in the controls. The incidences of germinal epithelial degeneration of the testis in all exposed groups of males were significantly greater than that in the controls.

There were increased incidences of myelofibrosis of the bone marrow in 12,000 ppm males (0 ppm, 3/50; 3,000 ppm, 8/50; 6,000 ppm, 8/50; 12,000 ppm, 19/49) and an increased severity but not incidence of this lesion in exposed females. There were also increased incidences of pigmentation of minimal to mild severity in the bone marrow of 6,000 and 12,000 ppm males (0/50, 2/50, 5/50, 16/49) and females (2/50, 3/50, 11/50, 11/50).

Also, the incidences of hematopoietic cell proliferation in the red pulp of the spleen (10/50, 22/50, 28/50, 21/49) in all exposed groups of males were significantly greater than that in the controls, and the severity of this lesion increased with increasing exposure concentration.

The incidences of hepatocellular adenoma in all exposed groups of males and females and of hepatocellular adenoma or carcinoma (combined) in 6,000 and 12,000 ppm males and all exposed groups of females were significantly less than those in the controls, and these lesions occurred with significant negative trends. Multiple hepatocellular adenomas were observed more frequently in the control groups than in the exposed groups. The incidences of clear cell and eosinophilic foci in all exposed groups of males and of mixed cell foci in 12,000 ppm males were significantly less than those in the controls. The incidences of eosinophilic foci in exposed groups of females were significantly less than in the controls.

GENETIC TOXICOLOGY

Phenolphthalein, tested in two laboratories, was not mutagenic in any of four strains of *Salmonella typhimurium* with or without S9 metabolic activation enzymes, and no induction of sister chromatid exchanges was observed in cultured Chinese hamster ovary cells treated with phenolphthalein with or without S9. However, significant increases in chromosomal aberrations were observed after treatment of cultured Chinese hamster ovary cells with phenolphthalein in the presence of S9, and the frequencies of micronucleated erythrocytes were increased in peripheral blood samples from male and female mice administered phenolphthalein in feed for 13 weeks.

CONCLUSIONS

Under the conditions of these 2-year feed studies, there was *clear evidence of carcinogenic activity** of phenolphthalein in male F344/N rats based on markedly increased incidences of benign pheochromocytomas of the adrenal medulla and of renal tubule adenomas and adenomas or carcinomas (combined). There was *some evidence of carcinogenic activity* of phenolphthalein in female F344/N rats based on the increased incidences of benign pheochromocytomas of the adrenal medulla in the 12,000 ppm group and of benign or malignant pheochromocytomas (combined) in the 12,000 and 25,000 ppm groups. There was *clear evidence of carcinogenic activity* of phenolphthalein in male B6C3F$_1$ mice based on increased incidences of histiocytic sarcomas and of malignant lymphomas of thymic origin. There was *clear evidence of carcinogenic activity* of phenolphthalein in female B6C3F$_1$ mice based on increased incidences of histiocytic sarcomas, malignant lymphomas of all types, lymphomas of thymic origin, and benign sex-cord stromal tumors of the ovary.

Exposure of rats to phenolphthalein in feed for 2 years resulted in increased incidences of focal hyperplasia of the adrenal medulla in males and in increased incidences and/or severity of nephropathy of the kidney in males and females. Exposure of mice to phenolphthalein in feed for 2 years resulted in increased incidences of atypical hyperplasia of the thymus in males and females, degeneration of the germinal epithelium of the testis in males, and ovarian hyperplasia in females.

Exposure of mice to phenolphthalein in feed for 2 years resulted in decreased incidences of hepatocellular neoplasms and nonneoplastic lesions in males and females.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 12. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 14.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Phenolphthalein

	Male F344/N Rats	Female F344/N Rats	Male $B6C3F_1$ Mice	Female $B6C3F_1$ Mice
Doses	0, 12,000, 25,000, or 50,000 ppm in feed (approximately 500, 1,000, or 2,000 mg/kg per day)	0, 12,000, 25,000, or 50,000 ppm in feed (approximately 500, 1,000, or 2,500 mg/kg per day)	0, 3,000, 6,000, or 12,000 ppm in feed (approximately 300, 600, or 1,200 mg/kg per day)	0, 3,000, 6,000, or 12,000 ppm in feed (approximately 400, 800, or 1,500 mg/kg per day)
Body weights	Exposed groups lower than the control group	Exposed groups lower than the control group	12,000 ppm group slightly lower than the control group	Exposed groups lower than the control group
2-Year survival rates	21/50, 15/50, 15/50, 13/50	30/50, 38/50, 32/50, 38/50	40/50, 33/50, 36/50, 36/49	39/50, 31/50, 34/50, 28/50
Nonneoplastic effects	Adrenal medulla: focal hyperplasia (13/50, 22/50, 18/50, 23/50) Kidney: severity of nephropathy (1.8, 2.9, 3.1, 3.1)	Kidney: nephropathy (34/50, 45/50, 43/50, 44/50); severity of nephropathy (1.2, 1.4, 1.5, 1.5)	Thymus: atypical hyperplasia (0/43, 3/46, 7/44, 7/42) Testis: germinal epithelial degeneration (1/50, 49/50, 50/50, 47/48)	Thymus: atypical hyperplasia (0/48, 7/44, 6/49, 5/45) Ovary: hyperplasia (4/50, 11/49, 10/50, 17/50)
Neoplastic effects	Adrenal medulla: benign pheochromocytoma (17/50, 34/50, 34/50, 34/50) Kidney: renal tubule adenoma (standard evaluation - 0/50, 4/50, 2/50, 6/50; extended evaluation - 1/50, 7/50, 15/50, 11/50; standard and extended evaluations combined - 1/50, 10/50, 15/50, 15/50); renal tubule adenoma or carcinoma (standard evaluation - 0/50, 5/50, 3/50, 7/50; extended evaluation - 1/50, 7/50, 15/50, 11/50; standard and extended evaluations combined - 1/50, 10/50, 16/50, 16/50)	Adrenal medulla: benign pheochromocytoma (3/50, 11/50, 9/50, 2/49); benign or malignant pheochromocytoma (3/50, 12/50, 10/50, 2/49)	All organs: histiocytic sarcoma (1/50, 3/50, 11/50, 12/49); malignant lymphoma (thymic origin) (0/50, 4/50, 7/50, 2/49)	All organs: histiocytic sarcoma (0/50, 2/50, 7/50, 7/50); malignant lymphoma (all types) (15/50, 28/50, 33/50, 25/50); malignant lymphoma (thymic origin) (1/50, 9/50, 10/50, 7/50) Ovary: benign sex-cord stromal tumor (0/50, 7/49, 6/50, 5/50)

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Phenolphthalein (continued)

	Male F344/N Rats	Female F344/N Rats	Male $B6C3F_1$ Mice	Female $B6C3F_1$ Mice
Decreased incidences	None	None	Liver: hepatocellular adenoma (22/50, 12/50, 8/50, 10/49); hepatocellular adenoma or carcinoma (27/50, 20/50, 11/50, 16/49); clear cell focus (24/50, 6/50, 1/50, 0/49); eosinophilic focus (22/50, 6/50, 1/50, 1/49); mixed cell focus (6/50, 2/50, 1/50, 0/49)	Liver: hepatocellular adenoma (17/50, 2/50, 6/50, 1/50); hepatocellular adenoma or carcinoma (21/50, 3/50, 6/50, 2/50); eosinophilic focus (20/50, 4/50, 2/50, 1/50)
Level of evidence of carcinogenic activity	Clear evidence	Some evidence	Clear evidence	Clear evidence

Genetic toxicology

Salmonella typhimurium gene mutations:	Negative in strains TA98, TA100, TA1535, and TA1537 with and without S9
Sister chromatid exchanges	
Cultured Chinese hamster ovary cells *in vitro*:	Negative with and without S9
Chromosomal aberrations	
Cultured Chinese hamster ovary cells *in vitro*:	Positive with S9; negative without S9
Micronucleated erythrocytes	
Mouse peripheral blood *in vivo*:	Positive

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence** and **some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that cannot be evaluated because of major flaws (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on phenolphthalein on December 5, 1995, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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National Cancer Institute
Frederick, MD

* Did not attend

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On December 5, 1995, the draft Technical Report on the toxicology and carcinogenesis studies of phenolphthalein received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J.K. Dunnick, NIEHS, introduced the toxicology and carcinogenesis studies of phenolphthalein by describing the uses of the chemical and rationale for the study, describing the experimental design, reporting on survival and body weight effects, and commenting on chemical-related neoplasms and nonneoplastic lesions in male and female rats and mice. Dr. Dunnick noted that molecular biology studies in collaboration with NIEHS intramural scientists are in progress or planned, including studies of the chemical in transgenic mouse models. Pharmacokinetic studies are in progress. Dr. J.R. Hailey, NIEHS, presented photomicrographs of lesions of the hematopoietic system, examples of histiocytic sarcoma, malignant lymphoma of thymic origin and associated hyperplasia, ovarian proliferative lesions, and testicular atrophy. The proposed conclusions were *clear evidence of carcinogenic activity* of phenolphthalein in male F344/N rats, *some evidence of carcinogenic activity* of phenolphthalein in female F344/N rats, and *clear evidence of carcinogenic activity* in male and female B6C3F$_1$ mice.

Dr. Goldsworthy, a principal reviewer, agreed with the proposed conclusions. He stated that the Technical Report should provide assurance that biological responses observed, especially those related to estrogenic effects, are attributable to phenolphthalein and not to lipophilic impurities. Small quantities of such impurities have been shown to account for the estrogenic activity in commercial preparations of the sulfonated analog of phenolphthalein, phenolsulfonphthalein or phenol red. Dr. Dunnick responded that the phenolphthalein used in these studies was 99% pure. She noted that Dr. M.D. Shelby, NIEHS, is developing assays for estrogenic activity and anticipated that the estrogenic potential of phenolphthalein could be compared with those of phenol red and of lipophilic impurities. Dr. Goldsworthy said there should be a comprehensive treatment of the estrogenic responses; for example, discussion on decreased incidences of liver lesions in mice. Dr. Dunnick proposed isolating the 1% impurity and examining its carcinogenic and estrogenic activity in short-term model systems such as transgenics and MCF-7 cells.

Dr. Ward, the second principal reviewer, was unable to attend the meeting but had submitted his review, which Dr. L.G. Hart, NIEHS, read into the record. Dr. Ward agreed with the proposed conclusions. He asked if the myelofibrosis reported was the typical lesion found in aging B6C3F$_1$ mice and probably estrogenic in origin. Dr. Hailey said that it was the lesion associated with aging.

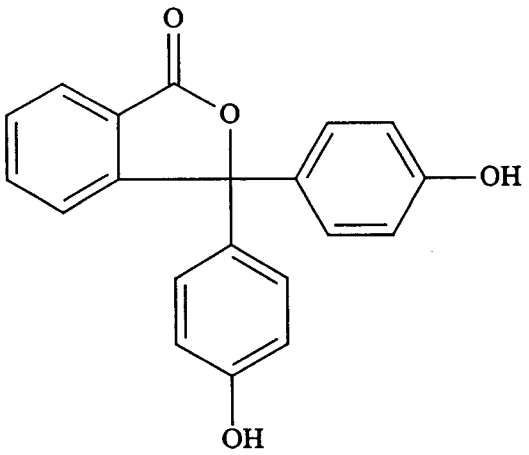
Dr. Taylor, the third principal reviewer, agreed with the proposed conclusions. He noted that pharmacokinetic data on phenolphthalein are rare in the literature and recommended that the pharmacokinetic data, particularly the lack of a dose-response relationship, in Appendix O be discussed in the body of the Technical Report. Dr. Dunnick said that these studies were limited, but that she would try to give more emphasis to the findings. Dr. Goldsworthy hoped that follow-up studies would look at phenolphthalein levels in organs such as the ovaries. Dr. G.W. Lucier, NIEHS, reported that follow-up pharmacokinetic studies will span a very wide dose range and will enable a better definition of the leveling off of tissue or blood levels of phenolphthalein in relation to dose.

Dr. Russo commented that there did not seem to be any influence of chemical treatment on pituitary gland or mammary gland lesions, with an even lower incidence of thyroid gland C-cell lesions than in control animals, all speaking against an estrogenic effect. Dr. Dunnick agreed, noting that another chemical with estrogenic activity, zearalenone, had not produced increases in mammary gland lesions in rats or mice. Dr. LeBoeuf asked whether the *in vitro* increases in chromosomal aberrations and *in vivo* increases in the frequency of micronucleated erythrocytes were consistent with other chemicals that may

have estrogen-like properties and can form quinones with metabolism, such as diethylstilbestrol. Dr. Shelby replied that the database is too small to reach any general conclusion. However, these increases along with the 2-year study results are fully consistent with the endocrine disrupting estromimetic compounds. Dr. W.T. Allaben, National Center for Toxicological Research and Food and Drug Administration (FDA), thanked the NTP, and particularly Dr. Dunnick, for sharing information during the study and review process and noted that the additional studies mentioned will be helpful to the FDA in its further review toward making an assessment regarding potential human risk.

Dr. Goldsworthy moved that the Technical Report on phenolphthalein be accepted with the revisions discussed and with the conclusions as written. Dr. Taylor seconded the motion, which was accepted with six yes votes and one abstention (Dr. LeBoeuf).

INTRODUCTION



PHENOLPHTHALEIN

CAS No. 77-09-8

Chemical Formula: $C_{20}H_{14}O_4$ Molecular Weight: 318.33

Synonyms: 3,3-Bis(4-hydroxyphenyl)-1(3H)-isobenzofuranone; 3,3-bis(*p*-hydroxyphenyl)phthalide; α-(*p*-hydroxyphenyl)-α-(4-oxo-2,5-cyclohexadien-1-ylidene)-*o*-toluic acid

Trade names: Agoral®, Alophen®, Colax®, Correctol®, Dialose®, Doxidan®, Espotabs®, Evac-U-Gen®, Evac-U-Lax®, Ex-Lax®, Feen-A-Mint®, FemiLax®, Kondremul®, LaxCaps®, Lax-Pills®, Medilax®, Modane®, Phenolax®, Prulet®

CHEMICAL AND PHYSICAL PROPERTIES

Phenolphthalein is a white or yellowish white, odorless, tasteless powder consisting of minute triclinic crystals (often twinned). It has a specific gravity of 1.277 at 32/4° C and a melting point range of 262° to 263° C (Weast, 1987) or 258° to 262° C (*Merck Index*, 1989), depending on the relative amounts of associated impurities. Although not flammable, phenolphthalein emits acrid smoke and fumes when heated to decomposition (*Sax's*, 1992). Phenolphthalein is readily soluble in alcohol (1 g dissolves in 12 to 15 mL) or ether (1 g dissolves in approximately 100 mL) and very slightly soluble in chloroform. It is almost insoluble in water; however, its solubility is increased in physiologic buffered solutions simulating intestinal contents, i.e., 7.8 mg/dL in Krebs-Ringer-bicarbonate solution, pH 7.4 (Sharaiha *et al.*, 1983). The solubility of aqueous phenolphthalein is also pH dependent and does not exceed 6 mg/dL without the addition of ethanol until the pH is above the physiologic range, i.e., greater than 9 (Fantus and Dyniewicz, 1937; Hubacher, 1945). Solutions containing phenolphthalein are colorless to pH 8.5 and pink to deep red above pH 9 (Figure 1). Phenolphthalein is used as a laboratory reagent and an acid-base indicator in titrations of mineral and organic acids and most alkalies (*Merck Index*, 1989).

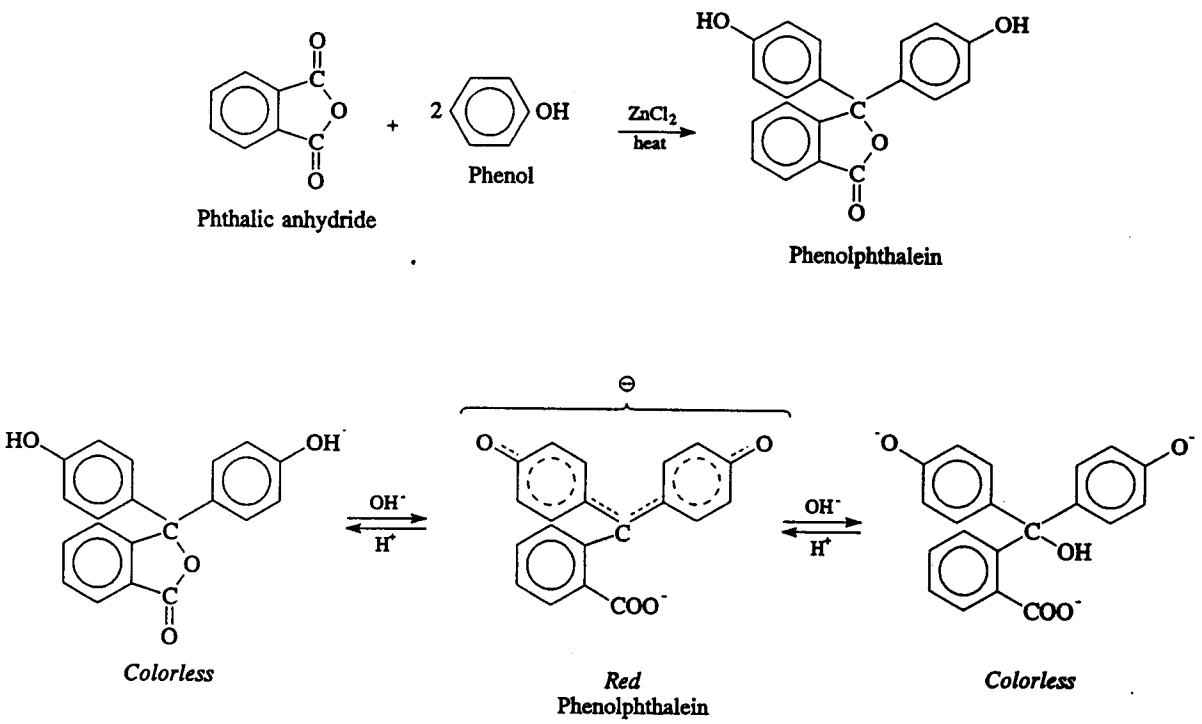


FIGURE 1
Phenolphthalein Synthesis and Reactions as an Indicator (Morrison and Boyd, 1966)

PRODUCTION, USE, AND HUMAN EXPOSURE

Phenolphthalein is synthesized commercially by the condensation of phthalic anhydride with phenol in the presence of a dehydrating agent (sulfuric acid) (Figure 1). After the mixture is heated at 120° C for 10 to 12 hours, the phenolphthalein residue is extracted with boiling water and then dissolved in dilute sodium hydroxide solution, filtered, and precipitated with acid (*Remington's*, 1990). Approximately 250 tons of phenolphthalein are produced in the United States annually by a single manufacturer, Sigma-Aldrich Corporation (*Chemical Economics Handbook*, 1992).

Early studies in humans assessing the safety of phenolphthalein when used as a pH-dependent coloring agent in adulterated wine led to recognition of its cathartic action (von Vámossy, 1908). Phenolphthalein is available worldwide in numerous proprietary over-the-counter laxative preparations including tablets, powders, suspensions, capsules, and chewables; the fact that phenolphthalein is tasteless makes it desirable for marketing in the form of chocolate candy or chewing gum (AHFS, 1995).

The usual daily oral laxative doses for white or yellow phenolphthalein are 30 to 270 mg for adults and children 12 years of age and older, 30 to 60 mg for children 6 to 11 years of age, and 15 to 30 mg for children 2 to 5 years of age, although the use of stimulant laxatives is generally not recommended in children younger than 6 years of age (*Fed. Regist.*, 1975; AHFS, 1995).

Uses of phenolphthalein as a laxative-cathartic may include: as bowel evacuants prior to bowel surgery

or radiologic, proctoscopic, and colonoscopic procedures; to alleviate pain of elimination from an episiotomy wound, anal fissures, perianal abscesses, or after surgery; to reduce intra-abdominal pressure during elimination in patients with hernias, anorectal stenosis, or abnormalities of the cerebral or coronary arterial vessels; to relieve constipation during pregnancy or the puerperium; to modify the effluent in ileostomy and colostomy patients; to compensate for lost abdominal and perineal muscle tone in geriatric patients with poor eating habits; and for altered bowel motility in patients receiving anticholinergic or narcotic therapy (Pietrusko, 1977; *Goodman and Gilman's*, 1990).

However, laxative-cathartics, which are habit forming (i.e., chronic usage results in dependence), are commonly used to self-medicate symptoms of constipation and are frequently abused in an effort to control weight (Pietrusko, 1977; *Goodman and Gilman's*, 1990). Ten percent of college students who participated in a 1981 survey admitted to purging behavior, i.e., laxative use and self-induced vomiting (Halmi *et al.*, 1981). Purging behavior is also associated with the eating disorders anorexia nervosa and bulimia nervosa; the prevalence of laxative abuse in patients with bulimia nervosa has been reported as 38% to 63% (Van Rooyen and Ziady, 1972; Pyle *et al.*, 1981; Johnson *et al.*, 1982; Bo-Linn *et al.*, 1983; Mitchell *et al.*, 1983; Fairburn and Cooper, 1984). A review by Cummings (1974) indicated that over 90% of chronic laxative abusers are women.

It is not possible to quantitate exposure to phenolphthalein via medication because of the large number of over-the-counter laxative preparations (Fleischer *et al.*, 1969; Van Rooyen and Ziady, 1972; Cummings *et al.*, 1974; LaRusso and McGill, 1975; Bytzer *et al.*, 1989). In the United States, Great Britain, and Australia, the rate of regular laxative use in populations who are apparently well is approximately 17% to 20% (Connell *et al.*, 1965; Dent *et al.*, 1986; Wu *et al.*, 1987; Kune, 1993). In 1975, 130 million dollars were spent in the United States on over-the-counter laxative preparations (Binder and Donowitz, 1975). In 1980, overall United States sales increased to approximately one billion dollars (Curry, 1990).

According to the National Occupational Exposure Survey, 75,243 workers (26% of whom were female) were potentially exposed to phenolphthalein in the years 1981 to 1983. Of the potentially exposed workers, 20,122 (65% female) were employed in the health services (NIOSH, 1990).

Regulatory Status

Based on a review of over-the-counter laxative-cathartics conducted by a Food and Drug Administration panel as part of a drug efficacy study implementation project, phenolphthalein was approved for human use and considered to be "generally recognized as safe" (*Fed. Regist.*, 1975).

Pharmacokinetics, Absorption, Distribution, Metabolism, and Excretion

Experimental Animals

Phenolphthalein is absorbed from the intestine and is excreted in the bile, urine, feces, and milk (Visek *et al.*, 1956; AHFS, 1995). Once phenolphthalein is absorbed, it is conjugated with glucuronic acid via uridine diphosphate glucuronosyltransferase (UDPGT; a phospholipid-dependent enzyme system) in the liver and intestine (Sund and Hillestad, 1982) and is distributed throughout the body in the blood and lymph (Visek *et al.*, 1956). Studies reported in the literature provide evidence for enzyme multiplicity of UDPGT. Steroidal and nonsteroidal UDPGT may have different membrane environments, and certain tissues such as the kidney and uterus have been shown to have a lower UDPGT activity for xenobiotic estrogenic compounds such as phenolphthalein (Lucier and McDaniel, 1977). Minor amounts of sulfate conjugate metabolites have also been detected in mucosal sheets isolated from the jejunum and colon of the guinea pig (Sund and Lauterbach, 1986).

Phenolphthalein conjugation enzyme activity (uridine diphosphatase glucuronosyltransferase) is absent or very low in microsomes from fetal or neonatal rat and guinea pig liver compared to activity from adult livers (Jondorf *et al.*, 1958; Wishart, 1978a). Glucuronide conjugation activity in adult rats is

stimulated by phenobarbital and unaffected by 3-methylcholanthrene (Wishart, 1978b).

Pharmacokinetic and tissue distribution studies in mice (strain not specified) and dogs (breed not specified) given 4.8 mg [^{14}C]-phenolphthalein/kg (uniformly labeled) indicated that phenolphthalein was widely and evenly distributed throughout the body, with levels of radioactivity parallel to the concentration in the blood. In dogs, approximately 50% of an oral dose was recovered in the feces and 36% in the urine after 72 hours (Visek *et al.*, 1956).

In the mouse studies (Visek *et al.*, 1956), no respiratory [^{14}C]-carbon dioxide was recovered following administration of phenolphthalein labeled with ^{14}C on the nonaromatic carbon of the lactone ring, indicating that the bonds to the labeled carbon atoms probably were not broken. Within 48 hours approximately 96% of the administered radioactivity was recovered in the urine and feces (56% in the urine and 38% in the feces following an oral dose; 30% in the urine and 68% in the feces following an intravenous dose). At 30 minutes to 6 hours, the highest levels of radioactivity appeared in the liver, gallbladder, and small intestine. On a total organ basis, the small intestine, which plays a major role in the excretion of phenolphthalein, contained more radioactive label than did the large intestine at all measurement intervals. However, when equated on a unit weight basis, the radioactivity of the large intestine and its contents increased substantially 6 hours after dosing.

Whole-body autoradiography studies in male BOM:NMRI mice given an intragastric dose of 1 mL/kg [^{14}C]-phenolphthalein (10 μCi/100 g) showed initial high levels of radioactivity in the stomach, gallbladder, and small intestine at 10 and 20 minutes. Radioactivity was observed in peripheral organs (including the kidney, liver, and skin) showing that the drug was absorbed from the gastrointestinal tract. The radiolabel moved along the intestinal tract, reaching the large intestine after 2 hours and showing maximum activity in the rectum after 4 hours. No radiolabel was detected in autoradiograms 2 days after dosing (Sund *et al.*, 1986).

Phenolphthalein undergoes extensive first pass metabolism in the intestinal epithelium and liver, which results in almost complete conversion of phenolphthalein to its glucuronide (Parker *et al.*, 1980). Studies by Colburn *et al.* (1979) demonstrated that 6 hours after intravenous administration of [^{3}H]-phenolphthalein to female Wistar rats, all radioactivity in the systemic circulation was present as the conjugate. In addition, a secondary peak in blood radioactivity occurred 5 to 6 hours after intravenous administration and coincided with the absorption of [^{3}H]-phenolphthalein from the intestine following bacterial ß-glucuronidase hydrolysis of [^{3}H]-phenolphthalein glucuronide excreted in the bile. Hydrolysis of phenolphthalein glucuronide to the aglycone is a rate-limiting step in enterohepatic recirculation (Bergan *et al.*, 1982); pretreatment with antibiotics to suppress intestinal microflora decreased the absorption of [^{3}H]-phenolphthalein from 85% to 22% in female Wistar rats following intraduodenal administration of [^{3}H]-phenolphthalein glucuronide (Parker *et al.*, 1980).

In a study with beagle dogs, two males were given 8- or 10-hour intravenous infusions of phenolphthalein at a rate of 177 μg/minute per kg body weight, and food was introduced at 2, 4, 6, and 8 hours during the study to stimulate gallbladder contraction. Steady-state serum levels were 7 μg/mL for phenolphthalein and 30 μg/mL for phenolphthalein glucuronide, and significant secondary peaks occurred for both compounds, an observation consistent with enterohepatic recirculation (Wilhelm *et al.*, 1992).

Phenolphthalein has been used as a model compound for enterohepatic recirculation studies in rats (Colburn *et al.*, 1979), and surgical cannulation of the bile duct has been used to evaluate the extent of enterohepatic recirculation in rats and dogs. In bile-duct-cannulated female Wistar rats, 95% of an intraperitoneal dose of 25 mg [^{3}H]-phenolphthalein/kg was recovered in the bile as the glucuronide within 24 hours, while only 0.2% was recovered in the urine. Administration of the same dose to intact rats resulted in recovery of 86% in the feces, predominantly as the parent drug, and 10% in the urine as the glucuronide (Parker *et al.*, 1980). In another study in female Wistar rats with biliary fistulae, phenolphthalein was completely eliminated in the bile (100%), almost entirely in the form of phenolphthalein glucuronide (98%) (Millburn *et al.*, 1967). The

plasma disappearance and biliary excretion kinetics of phenolphthalein glucuronide in the rat have been characterized by Mehendale (1990). Male Sprague-Dawley rats of the CR-1 strain were intravenously administered 3, 30, or 60 mg phenolphthalein or 3, 30, or 100 mg phenolphthalein glucuronide, and the femoral vein, artery, and common bile duct were cannulated. After administration of phenolphthalein, 99.5% of the dose was eliminated in the bile as phenolphthalein glucuronide with only trace quantities (0.5%) as phenolphthalein. Following administration of phenolphthalein glucuronide, phenolphthalein was undetectable in the bile. Biliary excretion was saturable at higher doses of both compounds.

Female dogs (breed not specified) given 4.8 mg [^{14}C]-phenolphthalein/kg excreted 51% and 36% of the administered radioactivity in the feces and urine within 72 hours after an oral dose and 54% and 37% in the feces and urine following an intravenous dose. Following surgical cannulation of the bile duct, recovery of orally administered radiolabel from the same dogs over a 72-hour period was: feces, 31%; urine, 38%; and bile, 22%. The corresponding figures following an intravenous dose were: feces, 11%; urine, 35%; and bile, 43% (Visek *et al.*, 1956).

The investigations of Visek *et al.* (1956) also demonstrated that radiolabeled phenolphthalein crossed the placenta of mice, resulting in recovery of radiolabel from fetal tissues at 6, 24, and 96 hours after administration that paralleled that of maternal blood. In the dog, however, analysis of blood and tissue samples of puppies born 50 hours after the mother received a 4.8 mg/kg oral dose indicated less than 0.03% of the administered dose in the liver and gallbladder and no radiolabel in the blood, which was interpreted by the authors as exceedingly limited passage of the drug across the placenta.

Humans

Phenolphthalein absorption in humans has been estimated to be 15% of an oral dose (*Goodman and Gilman's*, 1990). The absorbed compound is excreted primarily in the urine as phenolic-hydroxyglucuronide or sulfate conjugates, and the urine becomes pink or red if it is sufficiently alkaline. Some conjugated compound is also excreted in the feces via the bile, and the resulting enterohepatic recirculation probably contributes to prolongation of the laxative effect, a hypothesis supported by the observation that phenolphthalein is ineffective as a laxative in jaundiced patients or experimental animals with ligated common bile ducts (Steigmann *et al.*, 1938; *Goodman and Gilman's*, 1990).

Small doses of phenolphthalein in humans (30 to 60 mg) are excreted entirely as conjugated metabolites in urine or feces, while larger doses (300 mg) result in excretion of both the free and conjugated drug (Williams, 1959).

Diarrhea in infants may be caused by phenolphthalein usage by the mother during breast feeding (Tyson *et al.*, 1937).

PHARMACOLOGY

Following oral administration of phenolphthalein, evacuation usually occurs within 4 to 8 hours, and a single dose may produce laxation for several days (AHFS, 1995). The major site of cathartic action for phenolphthalein in humans is considered to be the large intestine with minor changes occurring in the small intestine (Saunders *et al.*, 1978; Sund, 1983). Phenolphthalein has choleretic properties and has been shown to accelerate bile secretions in male rats (Takeda and Aburada, 1981).

Phenolphthalein, a diphenylmethane derivative, belongs to the class of stimulant or irritant laxative-cathartics originally thought to stimulate peristalsis by irritation of the colonic mucosa (Binder, 1977). Current views of the mechanism of action of phenolphthalein indicate that it has a hydrophoric effect, which alters intestinal fluid and electrolyte movement and causes a net accumulation of luminal fluid and increased fluidity of the intestinal contents (Binder and Donowitz, 1975; Gaginella and Bass, 1978; Sund, 1983). This effect on the sodium pump, which has been demonstrated in an isolated rabbit ileal loop model *in vivo* using measurement of ^{24}Na flux, involves inhibition of Na^+ ion transport from the gut lumen to the plasma. Accumulation of Na^+ ions in the lumen results in secondary accumulation of anions (chiefly Cl^- and HCO_3^-) to maintain electroneutrality at a pH of approximately 8 with luminal water retention (Phillips *et al.*, 1965). Inhibition of intestinal water absorption has been demonstrated in

experimental animal models and in humans. An *in vivo* model using isolated jejunal, ileal, and colonic segments of the intestine of male Sprague-Dawley rats was used to demonstrate that phenolphthalein inhibited net water transport to the same extent in all regions of the intestine (Saunders *et al.*, 1978). A study of six ileostomy patients by the same investigators indicated that inhibition of water absorption in both the large and small intestine contributes to the laxative effect of phenolphthalein; 100 mg given four times a day increased the weight of ileostomy output by 30% and Na^+ output by 39%.

In addition to inhibition of active transport of sodium across the rabbit ileum and frog skin (Phillips *et al.*, 1965), phenolphthalein inhibited the sodium-dependent uptake of 3-methyl-D-glucose by the hamster small intestine *in vitro* (Adamič and Bihler, 1967) and reduced intestinal glucose absorption in the rat (Hart and McColl, 1967). Chignell (1968) investigated the effects of various purgative drugs on Na^+, K^+-adenosine triphosphatase activity in microsomes from the intestinal brush border of male Sprague-Dawley rats. Phenolphthalein was the most potent enzyme inhibitor of all the purgatives studied, blocking 70% of enzyme activity, which suggests that inhibition of sodium transport is a direct result of inhibition of Na^+, K^+-adenosine triphosphatase.

Phenolphthalein has been shown to stimulate prostaglandin biosynthesis in the colon of female Sprague-Dawley rats, with release of E-series prostaglandins into the gut lumen (Cohen, 1982). The extent of prostaglandin release correlated with the net water flux (laxative action) induced by the drug, and pretreatment with inhibitors of prostaglandin synthesis (indomethacin or aspirin) reduced the laxative effect in rats and mice (Beubler and Juan, 1978; Capasso *et al.*, 1984). In *in vitro* studies in male rat intestinal homogenates, phenolphthalein (314 μM) significantly increased the conversion of arachidonic acid, a prostaglandin precursor, to prostaglandins and 5-hydroxy-eicosatetraynoic acid in the colon and to leukotriene B_4 in the jejunum and colon. Similarly, in human colon homogenates, 100 μg phenolphthalein/mL increased conversion of arachidonic acid to prostaglandins and leukotriene B_4 (Capasso *et al.*, 1987).

Autore *et al.* (1984) attributed the laxative effect of a 16 mg/kg intragastric dose of phenolphthalein in male Wistar rats to stimulation of histamine and 5-hydroxytryptamine production and increased formation of prostaglandins, effects which were reduced by pretreatment with indomethacin. They concluded that the stimulation of biologically active amines may be due to altered gut motility, an overproduction of prostaglandin-like material, or a non-related direct effect of phenolphthalein. In a more recent study, there was an increase in kininogen content (measured as bradykinin equivalents/g wet tissue weight) in the colon of phenolphthalein-treated male Wistar rats, thus implicating kinins in the induction of laxation (Autore *et al.*, 1990). Augmentation of muscle contractions induced by prostaglandin E_2, demonstrated in the rat stomach and the longitudinal muscle of the guinea pig ileum and colon following *in vitro* administration of 10 μg phenolphthalein/mL, was suggested as a possible contributing factor in phenolphthalein laxation (Capasso *et al.*, 1988).

Although the laxative effect of phenolphthalein in monkeys is similar to that in humans, the dose required to induce laxation (25 mg/kg) is approximately 10 times that required for humans (Loewe and Hubacher, 1941).

TOXICITY

Experimental Animals

Phenolphthalein, given intravenously in dogs (breed not specified), was classified as relatively nontoxic in early studies (Abel and Rowntree, 1909). However, in rats it is regarded as moderately toxic by the intraperitoneal route, with an LD_{Lo} of 500 mg/kg (*Sax's*, 1992).

In oral studies, female mice (strain not specified) fed 5, 25, or 50 mg phenolphthalein/kg per day for 135 days showed no toxic manifestations or evidence of histopathologic changes in the liver, kidney, or gastrointestinal tract (Visek *et al.*, 1956).

Phenolphthalein, at doses of 25 and 50 μg/mL, caused cytotoxic effects in cultured Chang liver cells

characterized by decreased cell growth and increased anaerobic glycolysis, i.e., increased glucose consumption and lactate production (Nishikawa, 1981). Phenolphthalein has also been shown to inhibit growth of strains of anaerobic bacteria *in vitro* and cause leakage of potassium ions from both anaerobes and aerobes, findings consistent with the antibacterial properties of phenolic compounds (Bergan *et al.*, 1982; Sund, 1983).

Humans

Under normal conditions, phenolphthalein is regarded as nontoxic and safe for consumption, although therapeutic oral doses may occasionally produce abdominal discomfort, diarrhea, nausea, decreased blood pressure, faintness, and red urine and feces (AHFS, 1995). Serious side effects have been reported in cases of habitual phenolphthalein consumption under conditions of abuse (Cooke, 1977; Pietrusko, 1977); hypersensitivity reactions have been limited to susceptible or allergic individuals (Davies, 1985).

The primary reported organ for phenolphthalein toxicity is the intestine; indiscriminate use of phenolphthalein results in chronic constipation and laxative dependence, loss of normal bowel function, and bowel irritation. Habitual use for several years may cause a "cathartic colon," i.e., a poorly functioning, atonic dilation of the colon, especially of the right side, resulting in extensive bowel retention. The clinical condition, which resembles chronic ulcerative colitis both radiologically and pathologically, involves thinning of the intestinal wall and loss of the normal mucosal pattern of the terminal ileum (Cummings, 1974; Cooke, 1977; Pietrusko, 1977; AHFS, 1995).

Anecdotal cases of long-term use or overdose of phenolphthalein have been associated with abdominal pain, diarrhea, vomiting, electrolyte imbalance (hypokalemia, hypocalcemia, and/or metabolic acidosis or alkalosis), dehydration, malabsorption, protein-losing gastroenteropathy, steatorrhea, anorexia, weight loss, polydipsia, polyuria, cardiac arrhythmias, muscle weakness, prostration, and histopathologic lesions (Heizer *et al.*, 1968; Velentzas and Ikkos, 1971; Cummings, 1974; LaRusso and McGill, 1975; Pohl and Lowe, 1978; AHFS, 1995). Kidney, muscle, and central nervous system disturbances are thought to be due to electrolyte imbalance. Intestinal sodium and water loss stimulates compensatory renin production and secondary aldosteronism, leading to sodium conservation and potassium loss by the kidney (hypokalemia). Hypokalemia contributes to renal insufficiency, sometimes associated with rhabdomyolysis (Copeland, 1994).

Abuse of phenolphthalein-containing laxatives has been associated with gastrointestinal bleeding and iron deficient anemia (Weiss and Wood, 1982), acute pancreatitis (Lambrianides and Rosin, 1984), and multiple organ damage in cases of massive overdosages, including fulminant hepatic failure and disseminated intravascular coagulation (Sidhu *et al.*, 1989). Individual hypersensitivity reactions to phenolphthalein have also been reported to cause renal irritation, encephalitis, cardiac arrest, respiratory disturbances, and death (AHFS, 1995).

Phenolphthalein allergy is often manifested by cutaneous inflammatory reactions or fixed drug eruptions, i.e., solitary or multiple, well-defined, erythematous macules that may progress to vesicles and/or bullae. These lesions characteristically recur in the same location with each subsequent dose of phenolphthalein and generally leave residual hyperpigmentation that increases in intensity with each exposure; numerous melanin-containing dermal macrophages have been demonstrated in pigmented areas (Wyatt *et al.*, 1972; Davies, 1985; Stroud and Rosio, 1987; Zanolli *et al.*, 1993). In extreme cases, recurrences have involved progressively more severe lesions characterized as bullous erythema multiforme, with focal hemorrhage and necrosis and perivascular lymphocytic infiltration (Shelley *et al.*, 1972), and in one case report, toxic epidermal necrolysis (Kar *et al.*, 1986).

A review of 204 cases of phenolphthalein ingestion in children 5 years of age and younger reported to the Pittsburgh Poison Center over a 30-month period indicated that ingestion of 1 g or less was associated with minimal risk of developing dehydration caused by excessive diarrhea and resulting fluid loss (Mrvos *et al.*, 1991). However, despite the low acute toxicity profile documented by the Pittsburgh study, cases of fatal phenolphthalein poisoning of children have been reported; symptoms of pulmonary and

cerebral edema, multiple organ toxicity, and encephalitis were attributed to hypersensitivity reactions (Cleves, 1932; Kendall, 1954; Sarcinelli *et al.*, 1970). Repeated administration of phenolphthalein-containing laxatives to children has led to serious illnesses and multiple hospitalizations (Fleisher and Ament, 1977; Meadow, 1977; Devore *et al.*, 1982; Rosenberg, 1987; Sugar *et al.*, 1991; Ayass *et al.*, 1993).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

In a three-generation study involving 678 births, no drug-related teratogenic or reproductive deficits were observed in mice (strain not specified) fed approximately 250 mg phenolphthalein/kg per day in chocolate (Stockinger, 1965).

Phenolphthalein has weak estrogenic activity. In a study in immature Wistar rats, the minimum effective dose of phenolphthalein required to cause an estrogenic response (increase in glycogen content) in the uterus was 4 mg, compared to a 0.1 mg minimum effective dose of diethylstilbestrol (Bitman and Cecil, 1970). Subcutaneous injection of phenolphthalein also stimulated growth of the immature rat uterus in a similar study (Nieto *et al.*, 1990).

Humans

Phenolphthalein competed with estrogen for binding to cultured MCF-7 human breast cancer cells, with a relative binding affinity 10^{-4} that of estradiol, and stimulated cell growth as measured by DNA and protein assays. Acting as an estrogen agonist, phenolphthalein induced elevated levels of progesterone receptor in the MCF-7 cells. Growth stimulation by both phenolphthalein and estradiol was blocked by the anti-estrogen, 4-hydroxytamoxifen (Ravdin *et al.*, 1987). No other information related to the reproductive or developmental toxicity of phenolphthalein in humans has been reported in the literature.

CARCINOGENICITY

Experimental Animals

No definitive information regarding the carcinogenic potential of phenolphthalein in experimental animals was found in the literature. However, phenolphthalein phosphate was inactive as an inducer of tumors at the site of subcutaneous injections in mice when given once weekly for 13 weeks. Following an 8-month, treatment-free period, no tumors were observed when the experiment was terminated at 11 months with a 50% survival rate (10/20). The value of this study as an indicator of the carcinogenic potential of phenolphthalein was limited because the compound was tested at a single unspecified dose level (Haddow and Horning, 1960).

Humans

In a study conducted in Melbourne, Australia with 1,408 subjects, there was no statistically significant increased risk for colorectal cancer in phenolphthalein laxative users (Kune, 1993). The cases included in this study included all histologically confirmed new patients in "The Melbourne Colorectal Cancer Study" from April 1980 to April 1981. Community controls were age/sex frequency-matched with the clinical cases and were randomly selected from the same geographic area. In another study following 11,888 residents of a retirement community in California for 4.5 years, the association between laxative use and risk of colorectal cancer was not significant (Wu *et al.*, 1987). Information on other cancer sites was not reported.

GENETIC TOXICITY

The mutagenicity of phenolphthalein has been investigated and the data indicate that the chemical, although not mutagenic in bacteria, is capable of inducing chromosomal damage in mammalian cells *in vitro* and *in vivo*. Phenolphthalein did not induce DNA damage in repair-deficient strains of *Bacillus subtilis* (Kada *et al.*, 1972; Fujita *et al.*, 1976), nor was it mutagenic in *Salmonella typhimurium* (Bonin *et al.*, 1981; Mortelmans *et al.*, 1986). However,

blood samples obtained from the mice at the end of the 13-week toxicity study with phenolphthalein showed significant increases in micronucleated polychromatic erythrocytes and normochromatic erythrocytes in males and females (Dietz *et al.*, 1992). Efforts to confirm the genotoxicity of phenolphthalein in mice resulted in a series of experiments that investigated the influence of various experimental parameters (route of administration, frequency of dosing, duration of exposure, carrier vehicle) on the phenolphthalein-induced micronucleus response in mice (Witt *et al.*, 1995). It was concluded that phenolphthalein at relatively high doses (greater than or equal to 2,000 mg/kg per day for at least 2 days) administered either in feed or by gavage induced micronuclei in erythrocytes of $B6C3F_1$ mice and that this indication of chromosomal damage could be detected in either bone marrow or blood. Lower doses of phenolphthalein (120 mg/kg per day) were highly effective in inducing micronucleated erythrocytes when administered over a longer period of time (14 weeks) in Swiss (CD-1®) mice. Phenolphthalein also induced dose-related increases in chromosomal aberrations in cultured Chinese hamster ovary cells treated in the presence of induced rat liver S9 (Witt *et al.*, 1995). These *in vitro* results provide additional evidence of the genotoxicity of phenolphthalein.

STUDY RATIONALE

The National Cancer Institute nominated phenolphthalein for study because of its widespread use as a component of numerous over-the-counter laxative preparations, and the lack of adequate testing for carcinogenicity in experimental animals.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF PHENOLPHTHALEIN

Phenolphthalein was obtained from Air Products and Chemicals, Inc. (Allentown, PA), in one lot (127-7809) and from Pharmco Laboratories, Inc. (Titusville, FL), in two lots (P3186-D5 and P9189-J1). Lot 127-7809 was used during the 14-day studies, lot P3186-D5 was used during the 13-week studies, and lot P9189-J1 was used during the 2-year studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO) (Appendix J). Reports on analyses performed in support of the phenolphthalein studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

All lots of the chemical, a yellow powder, were identified as phenolphthalein by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy and by melting point. The purity of all lots was determined by elemental analyses, Karl Fischer water analysis, functional group titration, thin-layer chromatography, and high-performance liquid chromatography. For lot 127-7809, elemental analyses for carbon, hydrogen, and oxygen were in agreement with the theoretical values for phenolphthalein. Karl Fischer water analysis indicated 0.36% ± 0.02% water. Functional group titration indicated a purity of 98.8% ± 0.8%. Thin-layer chromatography by two systems indicated a major spot, a minor impurity, a trace impurity, and a slight trace impurity. High-performance liquid chromatography with one system revealed a major peak and five impurities with a combined area of 2.19% relative to the major peak area; high-performance liquid chromatography with a second system revealed a major peak and four impurities with a combined area of 2.28% relative to the major peak area. The overall purity of lot 127-7809 was determined to be greater than or equal to 98%. For lot P3186-D5, elemental analyses for carbon and hydrogen were in agreement with the theoretical values for phenolphthalein. Karl Fischer water analysis indicated 0.193% ± 0.004% water. Functional group titration indicated a purity of 99.5% ± 0.4%. Thin-layer chromatography indicated a major spot, a minor impurity, and a trace impurity by one system and a major spot and a trace impurity by a second system. High-performance liquid chromatography revealed a major peak and three impurities with a combined area of 1.4% relative to the major peak area, and major peak comparisons of lot P3186-D5 with lot 127-7809 indicated a purity of 100.3% ± 0.6% for lot P3186-D5 relative to lot 127-7809. The overall purity of lot P3186-D5 was determined to be greater than or equal to 98%. For lot P9189-J1, elemental analyses for carbon and hydrogen were in agreement with the theoretical values for phenolphthalein. Karl Fischer water analysis indicated 0.12% ± 0.05% water. Functional group titration indicated a purity of 99.9% ± 0.5%. Thin-layer chromatography indicated a major spot and a trace impurity by one system and a major spot by a second system. High-performance liquid chromatography revealed a major peak and three impurities with a combined area of 1.2% relative to the major peak area, and major peak comparisons of lot P9189-J1 with lot 127-7809 indicated a purity of 100.9% ± 0.2% for lot P9189-J1 relative to lot 127-7809. The overall purity of lot P9189-J1 was determined to be greater than or equal to 99%.

Stability studies of lot 127-7809 were performed by the analytical chemistry laboratory using high-performance liquid chromatography. These studies indicated that phenolphthalein was stable as a bulk chemical for 2 weeks when stored protected from light at temperatures up to 60° C. To ensure stability, the bulk chemical was stored protected from light at 25° C in sealed containers during the 13-week studies and was stored protected from light at or below 27° C in sealed containers during the 2-year studies.

Stability was monitored during the 13-week and 2-year studies using high-performance liquid chromatography. No degradation of the bulk chemical was detected.

Preparation and Analysis of Dose Formulations

The dose formulations for all studies were prepared weekly by mixing phenolphthalein with feed (Table J1). Stability studies of the 6,000 ppm dose formulation of lot 127-7809 were performed by the analytical chemistry laboratory using high-performance liquid chromatography. The stability of the dose formulation was confirmed for at least 2 weeks when stored at temperatures up to 25° C. Homogeneity and stability studies of the 500 ppm dose formulation of lot P3186-D5 were performed by the analytical chemistry laboratory using high-performance liquid chromatography. Homogeneity was confirmed and the stability of the dose formulation was confirmed for at least 3 weeks when stored protected from light at room temperature.

Periodic analyses of the dose formulations of phenolphthalein were conducted at the study laboratories using high-performance liquid chromatography. During the 13-week studies, the formulations were analyzed every 6 weeks (Table J2). During the 2-year studies, the formulations were analyzed approximately every 8 weeks (Table J3). All of the dose formulations analyzed during the 13-week studies were within 10% of the target concentrations. Of the dose formulations analyzed during the 2-year studies, 99% (122/123) were within 10% of the target concentrations; the one formulation out of specifications was remixed and was within 10% of the target concentration. During the 2-year studies, 89% (40/45) of the animal room samples were within 10% of the target concentrations. Results of periodic referee analyses performed during the 13-week studies by the analytical chemistry laboratory agreed with the results obtained by the study laboratory (Table J4).

14-Day Studies

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Harlan Industries (Indianapolis, IN), and animals were quarantined for 19 days. Before initiation of the studies, two male and two female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. Groups of five male and five female rats and mice were fed diets containing 0, 6,250, 12,500, 25,000, 50,000, or 100,000 ppm phenolphthalein. Feed and water were available *ad libitum*. Rats and mice were housed five per cage. Clinical findings were recorded daily for rats and mice. Feed consumption was recorded on days 7 and 14 and whenever the feed had to be replenished due to an inadequate feed supply. The animals were weighed initially, on day 7, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

At the end of the 14-day studies, a necropsy was performed on all rats and mice. Histopathologic examinations were performed on one male control rat, two female control rats, two male control mice, one female control mouse, and two males and one female from the 100,000 ppm rats and mice. Table 1 lists the tissues and organs examined.

13-Week Studies

The 13-week studies were conducted to evaluate the cumulative toxic effects of repeated exposure to phenolphthalein and to determine the appropriate exposures to be used in the 2-year studies.

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Farms (Germantown, NY). On receipt, the rats and mice were 4 weeks old. Animals were quarantined for 12 to 14 days (rats) or for 14 days (mice) and were 6 weeks old on the first day of the studies. Before initiation of the studies, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. At the end of the studies, serologic analyses were performed on five male and five female sentinel rats and on five male and five female control mice using the protocols of the NTP Sentinel Animal Program (Appendix M).

Groups of 10 male and 9 or 10 female rats and mice were fed diets containing 0, 3,000, 6,000, 12,000, 25,000, or 50,000 ppm phenolphthalein. Additional groups of 10 male and 10 female rats designated for clinical pathology evaluations were

fed diets containing 0, 3,000, 6,000, 12,000, 25,000, or 50,000 ppm phenolphthalein. Feed and water were available *ad libitum*. Rats were housed five per cage, and mice were housed individually. Clinical findings were recorded weekly for rats and mice. Feed consumption was recorded weekly. The animals were weighed initially, weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

On days 5 and 21, all clinical pathology group rats were anesthetized with carbon dioxide, and blood was collected from the retroorbital sinus for hematology and clinical chemistry analyses. At the end of the 13-week study, all core study rats were anesthetized with carbon dioxide, and blood was collected from the retroorbital sinus for hematology and clinical chemistry analyses. Blood for hematology determinations was placed in tubes containing potassium EDTA as the anticoagulant. Hematology evaluations for hematocrit, hemoglobin, erythrocyte and reticulocyte counts, mean cell volume, mean cell hemoglobin, and leukocyte counts and differentials were performed according to standard hematology methods using a Baker 7000 (Baker Instruments, Allentown, PA) hematology analyzer. Platelet counts were performed using a Baker Model 810 (Baker Instruments, Allentown, PA) platelet analyzer. In addition, erythrocyte and platelet morphology were evaluated microscopically. For clinical chemistry analyses, samples were collected in plastic centrifuge tubes. Serum samples were analyzed using a Centrifichem 400 (Baker Instruments, Allentown, PA). The hematology and clinical chemistry parameters measured are listed in Table 1.

At the end of the 13-week studies, samples were collected from rats (core study) and mice exposed to 0, 12,000, 25,000, or 50,000 ppm phenolphthalein for sperm morphology and vaginal cytology evaluations. The parameters evaluated are listed in Table 1. Methods used were those described in NTP's sperm morphology and vaginal cytology evaluations protocol (NTP, 1984a). For 7 consecutive days prior to the end of the studies, the vaginal vaults of the females were moistened with saline, if necessary, and aspirated samples of vaginal fluids and cells were transferred to slides, air dried, fixed, and stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous cells were determined to ascertain estrus cycle stage (i.e., diestrus, proestrus, estrus, or metestrus). Male animals were evaluated for sperm morphology, count, and motility. The right testis and right epididymis were isolated, weighed, and fixed and preserved for histopathology. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides, and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted in five fields per slide by two observers. Following completion of the sperm motility estimates, each right cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution. Samples were then removed for morphology evaluation, and the remainder were heat fixed at 65° C. Sperm density was determined microscopically using a hemacytometer. Four sperm morphology slides were prepared for each animal evaluated. An aliquot of killed sperm suspension was stained in a test tube, spread on a microscope slide, coverslipped, and examined. Results of reproductive tissue evaluations and estrous cycle characterization are given in Appendix I.

A necropsy was performed on all core study rats and on all mice. The brain, heart, right kidney, liver, lung, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, and stained with hematoxylin and eosin. A complete histopathologic examination was performed on control and 50,000 ppm groups of rats and mice. Additionally, the liver (females), lung (males), and ovaries of rats were examined to a no effect level; the bone marrow and spleen (males) of mice were examined to a no effect level. Table 1 lists the tissues and organs routinely examined.

2-YEAR STUDIES

Study Design

Groups of 50 male and 50 female rats were fed diets containing 0, 12,000, 25,000, or 50,000 ppm phenolphthalein. Groups of 50 male and 50 female mice were fed diets containing 0, 3,000, 6,000, or 12,000 ppm phenolphthalein.

Source and Specification of Animals

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Farms (Germantown, NY) for use in the 2-year studies. Rats were quarantined for 13 days (males) or 15 days (females) before the beginning of the study. Mice were quarantined for 12 days (males) or 13 days (females) before the beginning of the study. Five male and five female rats and mice were selected for parasite evaluation and gross observation of disease. Male and female rats and female mice were approximately 7 weeks old at the beginning of the studies; male mice were approximately 6 weeks old at the beginning of the study. Serology samples were collected from up to five male and five female rats and mice for viral screening before the beginning of the study. Serology samples were collected from up to five male and five female sentinel rats at 5, 9, 12, 17, 18, and 23 months and from up to three male and three female sentinel rats and up to five male and five female 25,000 ppm rats at the end of the study. Serology samples were collected from up to five male and five female sentinel mice at 5, 12, and 18 months and from up to five male and five female 6,000 ppm mice at the end of the study. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix M).

Animal Maintenance

Rats were housed five per cage and mice were housed individually. Feed and water were available *ad libitum*. Feed consumption was measured approximately every 4 weeks by cage. Cages and racks were rotated every 2 weeks. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix L.

Clinical Examinations and Pathology

All animals were observed twice daily. Clinical findings were recorded every 4 weeks, and body weights were recorded initially, weekly for the first 13 weeks, and every 4 weeks thereafter.

On the last day of the 2-year studies, blood was collected from the retroorbital sinus of three male and three female anesthetized rats and mice from each exposed group at five time points (6 and 9 a.m. and 1, 4, and 9 p.m.) for the determination of plasma concentrations of total phenolphthalein (free and conjugated). The phenolphthalein concentration was not measured in the plasma of control animals because it was not expected to be a normal constituent of plasma and was not detected in plasma from untreated animals during development of the analytical procedure. Dosed feed was available *ad libitum* during the collection period. The plasma was stored at −20° C or lower until analysis. Plasma samples were analyzed using a procedure developed during the single-dose toxicokinetic studies (Appendix O) for the analysis of concentrations of total phenolphthalein (free and conjugated). Plasma samples were treated with β-glucuronidase and sulfatase enzyme preparation. Solid-phase extraction was used to isolate phenolphthalein, and the samples were analyzed using high-performance liquid chromatography. The average plasma concentrations of total phenolphthalein and standard deviations were calculated. The logarithms of these values were plotted as a function of time. Results of analyses of plasma concentrations for total phenolphthalein at the end of the 2-year studies are given in Appendix H.

A complete necropsy and microscopic examination were performed on all rats and mice. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, and stained with hematoxylin and eosin for microscopic examination. For all paired organs (i.e., adrenal gland, kidney, ovary), samples from each organ were examined. For the extended evaluation of renal tubule proliferative lesions, kidneys of male and

female rats were step sectioned at 1 mm intervals, and five or six additional sections were obtained from each animal. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The microscopic slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist reviewed the adrenal gland and kidney of male and female rats; the seminal vesicle and spleen of male rats; the ovary of female rats; the bone, bone marrow, liver, thymus, lymph nodes, and spleen of male and female mice; the testis of male mice; and the ovary, pituitary gland, thyroid gland, and uterus of female mice.

The quality assessment report and the reviewed slides were submitted to the NTP Pathology Working Group (PWG) chairperson, who reviewed all tissues and addressed any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologist, or lesions of general interest were presented by the chairperson to the PWG for review. The PWG consisted of the quality assessment pathologist and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of dose groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Thus, the final diagnoses represent a consensus of quality assessment pathologists, the PWG chairperson, and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the diagnosed lesions for each tissue type were evaluated separately or combined according to the guidelines of McConnell *et al.* (1986).

STATISTICAL METHODS

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions as presented in Tables A1, A5, B1, B5, C1, C5, D1, and D5 are given as the number of animals bearing such lesions at a specific anatomic site and the number of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A3, B3, C3, and D3) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., harderian gland, intestine, mammary gland, and skin) before microscopic evaluation, or when neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed. Tables A3, B3, C3, and D3 also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm, i.e., the Kaplan-Meier estimate of the neoplasm incidence that would have been observed at the end of the study in the absence of mortality from all other competing risks (Kaplan and Meier, 1958).

Analysis of Neoplasm Incidences

The majority of neoplasms in these studies were considered to be incidental to the cause of death or not rapidly lethal. Thus, the primary statistical method used was logistic regression analysis, which assumed that the diagnosed neoplasms were discovered as the result of death from an unrelated

cause and thus did not affect the risk of death. In this approach, neoplasm prevalence was modeled as a logistic function of chemical exposure and time. Both linear and quadratic terms in time were incorporated initially, and the quadratic term was eliminated if the fit of the model was not significantly enhanced. The neoplasm incidences of exposed and control groups were compared on the basis of the likelihood score test for the regression coefficient of dose. This method of adjusting for intercurrent mortality is the prevalence analysis of Dinse and Lagakos (1983), further described and illustrated by Dinse and Haseman (1986). When neoplasms are incidental, this comparison of the time-specific neoplasm prevalences also provides a comparison of the time-specific neoplasm incidences (McKnight and Crowley, 1984).

In addition to logistic regression, other methods of statistical analysis were used, and the results of these tests are summarized in the appendixes. These methods include the life table test (Cox, 1972; Tarone, 1975), appropriate for rapidly lethal neoplasms, and the Fisher exact test and the Cochran-Armitage trend test (Armitage, 1971; Gart *et al.*, 1979), procedures based on the overall proportion of neoplasm-bearing animals.

Tests of significance included pairwise comparisons of each exposed group with controls and a test for an overall dose-related trend. Continuity-corrected tests were used in the analysis of neoplasm incidence, and reported P values are one sided. The procedures described in the preceding paragraphs were also used to evaluate selected nonneoplastic lesions. For further discussion of these statistical methods, refer to Haseman (1984).

Analysis of Nonneoplastic Lesion Incidences

Because all nonneoplastic lesions in this study were considered to be incidental to the cause of death or not rapidly lethal, the primary statistical analysis used was a logistic regression analysis in which nonneoplastic lesion prevalence was modeled as a logistic function of chemical exposure and time.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed and control groups in the analysis of continuous variables. Organ and body weight data, which have approximately normal distributions, were analyzed using the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Clinical chemistry, hematology, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel, and implausible values were eliminated from the analysis. Average severity values were analyzed for significance using the Mann-Whitney U test (Hollander and Wolfe, 1973). Because the vaginal cytology data are proportions (the proportion of the observation period that an animal was in a given estrous stage), an arcsine transformation was used to bring the data into closer conformance with a normality assumption. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for simultaneous equality of measurements across exposure concentrations.

Historical Control Data

Although the concurrent control group is always the first and most appropriate control group used for evaluation, historical control data can be helpful in the overall assessment of neoplasm incidence in certain instances. Consequently, neoplasm incidences from the NTP historical control database (Haseman *et al.*, 1984, 1985) are included in the NTP reports for neoplasms appearing to show compound-related effects.

Quality Assurance Methods

The 13-week and 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 2-year studies were

submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covering completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report were conducted. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, so all comments had been resolved or were otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of phenolphthalein was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium*, sister chromatid exchanges and chromosomal aberrations in cultured Chinese hamster ovary cells, and increases in the frequency of micronucleated erythrocytes in mouse peripheral blood. The protocols for these studies and the results are given in Appendix E.

The genetic toxicity studies of phenolphthalein are part of a larger effort by the NTP to develop a database that would permit the evaluation of carcinogenicity in experimental animals from the structure and responses of the chemical in short-term *in vitro* and *in vivo* genetic toxicity tests. These genetic toxicity tests were originally developed to study mechanisms of chemically induced DNA damage and to predict carcinogenicity in animals, based on the electrophilic theory of chemical carcinogenesis and the somatic mutation theory (Miller and Miller, 1977; Straus, 1981; Crawford, 1985).

There is a strong correlation between a chemical's potential electrophilicity (structural alert to DNA reactivity), mutagenicity in *Salmonella*, and carcinogenicity in rodents. The combination of electrophilicity and *Salmonella* mutagenicity is highly correlated with the induction of carcinogenicity in rats and mice and/or at multiple tissue sites (Ashby and Tennant, 1991). Other *in vitro* genetic toxicity tests do not correlate well with rodent carcinogenicity (Tennant *et al.*, 1987; Zeiger *et al.*, 1990), although these other tests can provide information on the types of DNA and chromosome effects that can be induced by the chemical being investigated. Data from NTP studies show that a positive response in *Salmonella* is currently the most predictive *in vitro* test for rodent carcinogenicity (89% of the *Salmonella* mutagens were rodent carcinogens), and that there is no complementarity among the *in vitro* genetic toxicity tests. That is, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. The predictivity for carcinogenicity of a positive response in bone marrow chromosome aberration or micronucleus tests is not yet defined.

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Phenolphthalein

14-Day Studies	13-Week Studies	2-Year Studies
Study Laboratory		
Cannon Laboratories, Inc. (Reading, PA)	Microbiological Associates, Inc. (Bethesda, MD)	TSI Mason Laboratories, Inc. (Worcester, MA)
Strain and Species		
F344/N rats $B6C3F_1$ mice	F344/N rats $B6C3F_1$ mice	F344/N rats $B6C3F_1$ mice
Animal Source		
Harlan Industries (Indianapolis, IN)	Taconic Farms (Germantown, NY)	Taconic Farms (Germantown, NY)
Time Held Before Studies		
19 days	Rats: 12 to 14 days Mice: 14 days	Rats: 13 days (males) or 15 days (females) Mice: 12 days (males) or 13 days (females)
Average Age When Studies Began		
Not available	6 weeks	7 weeks (male and female rats and female mice) or 6 weeks (male mice)
Date of First Dose		
4 June 1979	Rats: 28 April 1987 Mice: 30 April 1987	Rats: 13 March (males) or 15 March (females) 1991 Mice: 27 November (males) or 28 November (females) 1990
Duration of Dosing		
14 days	13 weeks	104 weeks (males) or 105 weeks (females)
Date of Last Dose		
17 June 1979	Rats: 28-29 July 1987 (core study) or 18 May 1987 (clinical pathology groups) Mice: 30-31 July 1987	Rats: 10 March (males) or 18-19 March (females) 1993 Mice: 24-25 November (males) or 3-4 December (females) 1992
Necropsy Dates		
Rats: 19-20 June 1979 Mice: 19 June 1979	Rats: 28-29 July 1987 Mice: 30-31 July 1987	Rats: 10 March (males) or 18-19 March (females) 1993 Mice: 24-25 November (males) or 3-4 December (females) 1992
Average Age at Necropsy		
Not available	19 weeks	110 weeks (male mice), 111 weeks (male rats), or 112 weeks (female rats and mice)
Size of Study Groups		
5 males and 5 females	10 males and 9 or 10 females	50 males and 50 females

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Phenolphthalein (continued)

14-Day Studies	13-Week Studies	2-Year Studies
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weight	Same as 14-day studies	Same as 14-day studies
Animals per Cage 5	Rats: 5 Mice: 1	Rats: 5 Mice: 1
Method of Animal Identification Ear tag	Ear punch and toe clip	Tail tattoo
Diet NIH-07 open formula meal diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed daily	Same as 14-day studies, changed weekly	Same as 14-day studies, changed twice weekly
Water Distribution Tap water (Reading municipal supply) via water bottles changed weekly, available *ad libitum*	Tap water (Bethesda municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available *ad libitum*, changed every 2 weeks	Tap water (Worcester municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available *ad libitum*, changed every 2 weeks
Cages Polycarbonate cages with stainless steel tops, changed twice per week	Polycarbonate suspended cages (Lab Products, Inc., Rochelle Park, NJ), changed twice per week	Polycarbonate, solid-bottom cages (Lab Products, Inc., Rochelle Park, NJ), changed 3 times per week (male rats), twice per week (female rats), or weekly (male and female mice)
Bedding Absorb-Dri® hardwood chips, changed twice per week	Beta-chips® heat-treated hardwood chips (Northeastern Products Corp., Warrensburg, NY), changed twice per week	Sani-chips® heat-treated hardwood chips (P.J. Murphy Forest Products, Montville, NJ), changed 3 times per week (male rats), twice per week (female rats), or weekly (male and female mice)
Cage Filters Reemay® spun-bonded polyester filter sheet (Andico, Birmingham, AL)	Spun-bonded polyester filter sheet (Snow Filtration Co., Cincinnati, OH), changed every 2 weeks	Nonwoven fiber filters (Snow Filtration, Cincinnati, OH), changed every 2 weeks
Racks Racks changed every 2 weeks	Stainless steel racks (Lab Products, Inc., Rochelle Park, NJ), changed every 2 weeks	Same as 13-week studies

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Phenolphthalein (continued)

14-Day Studies	13-Week Studies	2-Year Studies
Animal Room Environment		
Temperature: 22° to 24° C Relative humidity: 45% to 55% Fluorescent light: 12 hours/day Room air: 10 to 15 changes/hour	Temperature: 21° to 23° C Relative humidity: 50% to 67% Fluorescent light: 12 hours/day Room air: minimum of 10 changes/hour	Temperature: 21° to 23° C Relative humidity: 37% to 58% Fluorescent light: 12 hours/day Room air: 10 changes/hour
Doses		
0, 6,250, 12,500, 25,000, 50,000, or 100,000 ppm in feed, available *ad libitum*	0, 3,000, 6,000, 12,000, 25,000, or 50,000 ppm in feed, available *ad libitum*	Rats: 0, 12,000, 25,000, or 50,000 ppm in feed, available *ad libitum* Mice: 0, 3,000, 6,000, or 12,000 ppm in feed, available *ad libitum*
Type and Frequency of Observation		
Observed once daily; animals were weighed initially, on day 7, at the end of the studies; clinical findings were recorded daily. Feed consumption was recorded by cage on days 7 and 14 and whenever the feed had to be replenished due to an inadequate feed supply.	Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; clinical findings were recorded weekly. Feed consumption was recorded weekly by cage.	Observed twice daily; animals were weighed initially, weekly for 13 weeks, monthly thereafter, and at the end of the studies; clinical findings were recorded every 4 weeks. Feed consumption was recorded approximately monthly by cage.
Method of Sacrifice		
Asphyxiation with carbon dioxide	Asphyxiation with carbon dioxide	Asphyxiation with carbon dioxide
Necropsy		
Necropsy was performed on all animals.	Necropsy was performed on all core study rats and on all mice. Organs weighed were brain, heart, right kidney, liver, lung, right testis, and thymus.	Necropsy was performed on all animals.
Clinical Pathology		
None	Blood was collected from the retroorbital sinus of rats in the clinical pathology groups on days 5 and 21 and of core study rats at the end of the study for hematology and clinical chemistry. ***Hematology:*** hematocrit, hemoglobin, erythrocyte and reticulocyte counts, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, platelet count, and total leukocyte counts and differentials. ***Clinical chemistry:*** urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and bile acids.	None

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Phenolphthalein (continued)

14-Day Studies	13-Week Studies	2-Year Studies
Histopathology		
Complete histopathology was performed on one male rat and two female rats from the control groups, on two male mice and one female mouse from the control groups, and on two males and one female from the 100,000 ppm rats and mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal glands, bone marrow, brain, ears (external and middle), esophagus, eyes, gallbladder (mice), heart, large intestine (colon and rectum), small intestine (duodenum, jejunum, and ileum) kidneys, larynx, liver, lungs (and mainstem bronchi), lymph nodes (mandibular and mesenteric), mammary gland, nose, ovaries, pancreas, parathyroid glands, pituitary gland, prostate gland, rib (and costochondral junction), salivary gland, skin, spinal cord (and sciatic nerve), spleen, stomach, testes (with seminal vesicle), thigh muscle, thymus, thyroid glands, trachea, urinary bladder, and uterus.	Complete histopathology was performed on 0 and 50,000 ppm rats and mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, brain, clitoral gland (rats), esophagus, femur (including marrow), heart, gallbladder (mice), large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidney, liver, lungs (and mainstem bronchi), lymph nodes (mandibular and mesenteric), mammary gland, nose, ovaries, pancreas, parathyroid gland, pituitary gland, preputial gland (rats), prostate gland, salivary gland, skin, spleen, stomach (including forestomach and glandular stomach), testes (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus. In rats, the liver (females), lung (males), and ovaries were examined to a no effect level. In mice, the bone marrow and spleen (males) were examined to a no effect level.	Complete histopathology was performed on all rats and mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal glands, brain (3 sections), clitoral gland, esophagus, femur (including marrow), heart, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidneys, liver, lung (and mainstem bronchi), lymph nodes (mandibular and mesenteric), mammary gland, nose (3 sections), ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary glands, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus.
Sperm Morphology and Vaginal Cytology Evaluations		
None	At the end of the study, sperm samples were collected from male rats (core study) and male mice in the 0, 12,000, 25,000, and 50,000 ppm groups for sperm morphology evaluations. The parameters evaluated included sperm density, morphology, and motility. The right cauda, right epididymis, and right testis were weighed. Vaginal fluid samples were collected for up to 7 consecutive days prior to the end of the studies from female rats (core study) and female mice in the 0, 12,000, 25,000, and 50,000 ppm groups. The parameters evaluated were relative frequency of estrous stages and estrous cycle length.	None
Determinations of Total Phenolphthalein in Plasma		
None	None	On the last day, blood was taken from three animals from each exposed group at 6 and 9 a.m. and 1, 4, and 9 p.m. for determination of plasma concentrations of total phenolphthalein.

DISCUSSION AND CONCLUSIONS

Phenolphthalein was nominated by the National Cancer Institute for toxicity and carcinogenicity studies because it is an over-the-counter laxative for which no carcinogenesis bioassay has been reported in the literature. Phenolphthalein is usually taken as a 60 mg oral dose, four times a day, for an approximate daily dose of 4 to 5 mg/kg. The probable mechanism for the laxative effect is stimulation of the mucosal nerve plexus in the colon, combined with water and electrolyte secretion or inhibition of water absorption in both large and small intestines, and decreased glucose absorption (Pietrusko, 1977; Saunders *et al.*, 1978; *Goodman and Gilman's*, 1990).

In the 13-week feed studies, rats and mice were exposed to phenolphthalein at concentrations up to 50,000 ppm. All rats and mice survived to the end of the studies. No chemical-related clinical signs of toxicity were observed in rats or mice, and there was no evidence of a laxative effect in these animals. In the 13-week studies, there were no chemical-related gross or microscopic lesions in rats. However, hypoplasia of the bone marrow occurred in male and female mice exposed to 12,000 ppm or greater. This lesion was characterized by decreased amounts of hematopoietic tissue with a decreased myeloid to erythroid ratio. Splenic hematopoiesis was observed in male mice exposed to 25,000 or 50,000 ppm. There were also effects on the male reproductive system in mice. There was no evidence of reproductive toxicity in female B6C3F_1 mice or in male or female F344/N rats. Lower epididymal weights and sperm density and an increased incidence of abnormal sperm were observed in male mice at all exposure concentrations evaluated (12,000, 25,000, and 50,000 ppm). In some seminiferous tubules one or more generations of germ cells in portions of or in the entire tubule were missing. Often such tubules were surrounded by seminiferous tubules that looked normal. This suggested an intermittent commitment of spermatogonia to differentiation, producing a "blank space" in the progression of cells through spermatogenesis. The lesion may be mimicked by dosing with a toxicant for a limited period of time followed by quick recovery of the testis, as has been observed in rats administered 2-methoxyethanol in drinking water (NTP, 1993a). However, this is the only instance in the history of the NTP where continued exposure to a chemical has apparently caused stops and starts in spermatogenesis.

In order to further analyze the reproductive toxic effects in mice, a continuous breeding study of phenolphthalein was conducted in Swiss (CD-1®) mice according to a protocol previously described (Lamb, 1985; Heindel *et al.*, 1989). In this study, male and female mice were given 1,000, 7,000, or 30,000 ppm phenolphthalein in feed for a 98-day cohabitation period, and fertility during this period was assessed (Appendix N; NTP, 1991). Phenolphthalein caused lower fertility in the 7,000 and 30,000 ppm groups. Overall, the mean numbers of litters per pair in the 7,000 and 30,000 ppm groups were 24% and 50% lower than in the controls. The numbers of live pups per litter in these groups were 58% and 59% lower than in the controls. There was also postnatal toxicity in the F_1 generation, as indicated by a 30% to 71% lower survival than that of the controls (all of the deaths occurred during the first 4 days of life).

Phenolphthalein can cross the placental barrier in mice (strain not specified) (Visek *et al.*, 1956). The lower survival in the F_1 generation observed in this continuous breeding study in Swiss (CD-1®) mice may be attributed to effects to the developing fetus from *in utero* exposure, postnatal exposure via the milk, or from a combination of these exposures. No reproductive or teratogenic effects were observed in mice (strain not specified) exposed to 250 mg phenolphthalein/kg body weight per day in feed (Stockinger, 1965), but this study was conducted at lower doses than those used in the continuous breeding study. In the continuous breeding study, the exposure concentrations of 7,000 and 30,000 ppm were equivalent to daily doses of approximately 1,056 and 4,530 mg/kg, respectively.

In a crossover mating trial with Swiss (CD-1®) mice (control males mated to 7,000 ppm females or control females mated to 7,000 ppm males), it was found that the lower fertility, as measured by a lower number of pups born per litter, resulted from effects in the female mice. It should be noted that the 7,000 ppm male Swiss (CD-1®) mice exhibited approximately a 20% lower sperm count relative to the controls, but the lower sperm count did not affect fertility. The relationship between lower sperm counts and fertility in a number of species is an area of continuing investigation (Medical Research Council, 1995).

In summary, phenolphthalein produced significant reproductive toxicity in both Swiss (CD-1®) and $B6C3F_1$ mice as measured by lower epididymal weight and sperm density. Exposed female Swiss (CD-1®) mice mated to control male Swiss (CD-1®) mice had fewer than half the number of live pups per litter as controls.

The mechanism for the reproductive toxicity observed in mice (but not in rats) is not fully understood. Phenolphthalein contains a triphenylmethane structure, and this structure may act as a nonsteroidal estrogen agonist or antagonist through interaction with the estrogen receptor protein (Ravdin *et al.*, 1987). Studies by other groups are underway to assess whether or not environmental estrogenic compounds play a significant role in reproductive disorders in humans (Jobling *et al.*, 1995; Medical Research Council, 1995).

In the 2-year feed studies of phenolphthalein, there were no chemical-related effects on survival in male or female rats or male mice; there was marginally lower survival in the 12,000 ppm female mice. No chemical-related clinical signs were observed.

Mean body weights of exposed groups of rats were 5% to 10% lower than those of the controls during most of the study, but toward the end of the study, the body weight differences became more pronounced. The body weight differences in mice were less than those in rats.

Feed consumption by exposed groups was similar to that by the controls. Comparisons of doses based on body surface area are provided in Table 21.

At the end of the 2-year study, organ toxicity and/or carcinogenic effects were observed in the adrenal gland of rats. These effects developed late in the study, and no toxicity was observed in these organs in the 13-week rat study.

The incidences of benign pheochromocytoma of the adrenal medulla were significantly increased in all groups of exposed male rats and in 12,000 ppm female rats. The incidences of benign pheochromocytoma in all groups of exposed male rats and in 12,000 and 25,000 ppm female rats exceeded the ranges in historical controls in NTP 2-year feed studies. The incidence of benign or malignant pheochromocytoma (combined) of the adrenal medulla in 12,000 and 25,000 ppm female rats was significantly greater than that of the controls and exceeded the range in historical controls in NTP 2-year feed studies. In the NTP database of approximately 450 chemicals, a chemical-induced neoplasm response is observed in the adrenal gland of rats two to three times more frequently than in mice. These increases in the incidences of pheochromocytoma of the adrenal medulla were considered to be related to exposure to phenolphthalein because the effect was observed in males and females, and in the groups cited above the neoplasm rates were greater than those of the concurrent and historical control rates. However, increased incidences were not observed in 50,000 ppm female rats.

The incidence of chronic nephropathy, which is common in aging rats (particularly males), was increased in exposed groups of female rats, and the severity of nephropathy was increased in exposed groups of male rats and slightly increased in exposed groups of female rats. In males, changes secondary to the exacerbated renal disease and indicative of compromised renal function (hyperplasia of the parathyroid gland, fibrous osteodystrophy of the bone, and mineralization and degeneration of the glandular stomach) were also increased, but not in a dose-related manner. Additionally, there were increases in the incidences of hyperplasia and neoplasms of the renal tubule epithelium. These lesions were confirmed by an extended evaluation of the kidneys. No mechanism by which these renal proliferative lesions developed was readily apparent, nor were these studies designed to determine a mechanism. One possible mode of action is consistent

TABLE 21
Summary of Feed and Compound Consumption by Rats and Mice in the 2-Year Feed Studies of Phenolphthalein

	0 ppm	12,000 ppm	25,000 ppm	50,000 ppm
Male Rats				
Mean for Weeks 1-13				
(g feed/day)	16.3	16.2	17.0	16.6
(mg/kg per day)	0	789	1,714	3,375
(mg/m^2 per day)[a]	0	4,102	8,912	17,550
Mean for Weeks 53-104				
(g feed/day)	14.4	15.3	15.9	15.8
(mg/kg per day)	0	434	953	1,880
Female Rats				
Mean for Weeks 1-13				
(g feed/day)	10.7	10.4	10.4	11.0
(mg/kg per day)	0	787	1,655	3,423
(mg/m^2 per day)	0	4,092	8,603	17,799
Mean for Weeks 53-105				
(g feed/day)	11.4	11.3	11.4	11.8
(mg/kg per day)	0	477	1,013	2,125

	0 ppm	3,000 ppm	6,000 ppm	12,000 ppm
Male Mice				
Mean for Weeks 1-13				
(g feed/day)	4.3	4.3	4.4	4.2
(mg/kg per day)	0	453	940	1,802
(mg/m^2 per day)	0	1,359	2,820	5,406
Mean for Weeks 53-104				
(g feed/day)	4.8	4.8	4.9	4.8
(mg/kg per day)	0	291	589	1,181
Female Mice				
Mean for Weeks 1-13				
(g feed/day)	5.1	4.9	5.3	5.1
(mg/kg per day)	0	651	1,407	2,640
(mg/m^2 per day)	0	1,953	4,221	7,920
Mean for Weeks 53-105				
(g feed/day)	5.3	5.3	5.2	5.2
(mg/kg per day)	0	311	617	1,255

[a] Calculation for body surface area dose based on Freireich *et al.*, 1966; $mg/m^2 = K_m \times$ (dose in mg/kg), where K_m is 37 for humans, 5.2 for rats, and 3.0 for mice. (K_m is a conversion based on average height-to-body weight ratio.) Compound consumption values for humans = 5 mg/kg and 185 mg/m^2.

with the theory of increased cell replication providing a "fertile ground" for increased mutation rates and neoplasm development. To a certain point, increased kidney damage is thought to increase the amount of renal tubule epithelial regeneration via cell replication. In one study, [^{3}H]-thymidine labeling demonstrated increased levels of DNA synthesis to be directly proportional to increased severity of nephropathy in aging female F344/NCr rats (Konishi and Ward, 1989).

The findings in the male rat kidney are similar to those observed with other chemicals including quercetin (NTP, 1992), coumarin (NTP, 1993b), and 3,4-dihydrocoumarin (NTP, 1993c). With these chemicals, there was no evidence for toxicity to the kidney in the 13-week studies, but toward the end of the 2-year studies, the severity of nephropathy increased in male rats (and to a lesser extent in female rats), and there were a few kidney neoplasms in male rats. The greater sensitivity of the male rat to this kidney toxicity is apparently due to a greater susceptibility of male rats to spontaneous nephropathy during aging and the exacerbation of this disease by chemical administration. Changes in glomerular permeability, resulting in proteinuria, progressive glomerular sclerosis, tubule damage, inflammation, and interstitial fibrosis, are associated with the process of aging in rats.

There was a decrease in the incidence of mammary gland fibroadenomas in groups of exposed female rats. Previous studies have shown that decreases in the incidences of some naturally occurring benign neoplasms, such as mammary gland fibroadenoma, are associated with lower body weights relative to those of the controls (Rao *et al.*, 1987; Seilkop, 1995). The lower incidences of female rat mammary gland neoplasms in this study were also thought to be related to the lower body weights.

At the end of the 2-year mouse study, carcinogenic effects were observed in the hematopoietic system of males and females and the ovary of females. There were increases in the incidences of histiocytic sarcomas and malignant lymphomas of thymic origin in all exposed groups of male and female mice. These effects were clearly related to exposure to phenolphthalein. The incidences of histiocytic sarcoma in all groups of exposed male and female mice exceeded the ranges in historical controls in NTP 2-year feed studies. The incidences of malignant lymphoma (all types) in all exposed groups of female mice also exceeded the ranges in historical controls in NTP 2-year feed studies.

Chemical-associated increases in the incidences of histiocytic sarcoma are uncommon in NTP studies; however, increased incidences have been observed in the studies with 1,3-butadiene (NTP, 1984b, 1993d) (an increase in thymic lymphomas also occurred) and tetrafluoroethylene (NTP, 1996a). Spontaneous histiocytic sarcoma occurs two to three times more frequently in female mice than in male mice. Similarly, in the tetrafluoroethylene mouse study, incidences in groups of females were about twice those observed in groups of males. The reverse was true in the current study. Histiocytic sarcomas occur most commonly in the liver, but are often widespread and involve numerous tissues. In the current study, histiocytic sarcoma was identified in the liver of all but three mice diagnosed with histiocytic sarcoma. Histiocytic sarcomas are generally considered to arise from a macrophage/histiocyte including the specialized Kupffer cell of the liver, but definitive data relative to the site of origin are lacking. In studies where histiocytic sarcomas were transplanted, the local lesions generally remained small, while the liver became quite large (Frith *et al.*, 1980). There is also evidence that many of these neoplasms produce excessive amounts of lysozyme, which can result in the accumulation of hyaline droplets in the proximal renal tubules as apparently occurred in the current study.

Exposure to phenolphthalein was associated with a clear increase in the incidence of malignant lymphoma (all types) in groups of exposed female mice. However, significant increased incidences of proliferative lymphocytic lesions that arose within the thymus were observed in both male and female mice. A number of classification schemes for malignant lymphomas (Dunn, 1954; Pattengale and Taylor, 1983; Wogan, 1984), primarily based upon histomorphology, have been proposed. However, it is often difficult to categorize consistently the various lymphomas. Further, as more is learned, it appears that there is little biological relevance for some of the classification schemes. Therefore, in most recent NTP studies, all lymphomas have been combined

under the diagnosis of malignant lymphoma. However, under certain circumstances, subclassification of lymphomas may be necessary to better understand the effects associated with an administered agent. In the $B6C3F_1$ mouse, the vast majority of spontaneous lymphomas arise from within the spleen or lymph nodes and are of B-cell origin. Lymphomas of the T-cells are much less common, and lymphomas arising from the thymus are uncommon. With phenolphthalein, 1,3-butadiene, and 2′,3′-dideoxycytidine (ddC) (Sanders *et al.*, 1995), the lymphomas originated primarily in the thymus. In this study, many proliferative lesions (atypical hyperplasias and lymphomas) clearly arose within the thymus of exposed males and females. Lymphomas were considered of thymic origin when they were observed only in the thymus or in the thymus and metastatic only to other sites within the chest cavity. Additionally, there was an increase in the incidence of atypical hyperplasia of the thymus in exposed males and females. It was apparent that, in general, the proliferative lesions of the thymus represented a morphological continuum in which the latter stage is malignant lymphoma. While there is a high probability that the lesions diagnosed as lymphomas are malignant neoplasms, there is less certainty as to the biological behavior of the smaller lesions (atypical hyperplasia). These smaller proliferative lesions are at least pre-neoplastic lesions and at most an early lymphoma. Many of the mice in this study had advanced lymphoma involving multiple tissues and organs. In those mice, there was less confidence in determining the site of origin; however, because of the numerous thymic lesions observed in this study, it was expected that a number of those widespread lymphomas were of thymic origin. Proliferative lesions (atypical hyperplasia and malignant lymphoma) of the thymus in exposed males and females were considered related to phenolphthalein exposure.

Phenolphthalein, 1,3-butadiene, and ddC are genotoxic in *in vivo* tests, suggesting that genotoxicity may be a contributing factor to the observation of thymic lymphomas. However, further work is in progress to see if phenolphthalein accumulates in the thymus and to identify possible mechanisms. Although mutations that activate *ras* proto-oncogenes are common in thymic lymphomas induced by a number of environmental agents including *N*-methylnitrosurea (Warren *et al.*, 1990; Corominas *et al.*, 1991) and radiation (Janowski *et al.*, 1990), *ras* mutations have not been commonly observed in the ddC-induced thymic lymphomas (Wiseman *et al.*, 1994) and were observed in only 2 of 11 thymic lymphomas from mice exposed to 1,3-butadiene (Goodrow *et al.*, 1994). *Ras* mutations were more common in 1,3-butadiene-induced lung and liver tumors than in the 1,3-butadiene-induced thymic lymphomas examined (6/7 lung tumors had K-*ras* mutations; 3/7 liver tumors had K-*ras* mutations; and 4/7 liver tumors had H-*ras* mutations) (Goodrow *et al.*, 1994).

In mice, exposure to phenolphthalein increased the incidence of ovarian hyperplasia in 3,000 and 12,000 ppm females and ovarian neoplasms in all groups of female mice. Generally, ovarian neoplasms fall into three categories: 1) epithelial, 2) germ cell, or 3) sex-cord stromal (e.g., granulosa cell tumors and luteomas). In the $B6C3F_1$ mouse, ovarian neoplasms are uncommon; epithelial cell tumors are slightly more common than granulosa cell tumors in control females. The morphology of the proliferative lesions of the ovary in the female mice in this study was somewhat distinctive from those typically observed in controls. It was apparent that cells composing proliferative lesions were often luteinized and were of sex-cord stromal origin. However, it was uncertain if component cells were granulosa, thecal, interstitial, or a combination. Non-specific categorization of these ovarian neoplasms as sex-cord stromal reflects that uncertainty.

Ovarian tumors (granulosal and epithelial) have previously been reported in mice in NTP studies after administration of eight other chemicals: benzene (NTP, 1986a), 1,3-butadiene (NTP, 1984b, 1993d), *N*-methylolacrylamide (NTP, 1989a), 5-nitroacenaphthene (NCI, 1978), nitrofurantoin (NTP, 1989b), nitrofurazone (NTP, 1988), 4-vinylcyclohexene (NTP, 1986b), and 4-vinyl-1-cyclohexene diepoxide (NTP, 1989c). The mechanism for the induction of ovarian tumors with some of these chemicals may involve atrophy of the ovary and oocyte destruction followed by a subsequent decrease in estrogen production, which leads to a compensatory increase in pituitary gland gonadotropin release. The increased stimulation by gonadotropins is thought to stimulate cell proliferation in the ovary and to promote the eventual development of

tumors. In the current study, no atrophy was observed, and the tumors are not similar to the epithelial and granulosa cell tumors observed in the previous NTP studies. The observation that phenolphthalein interacts with estrogen receptors (Ravdin *et al.*, 1987) may be one means by which this chemical stimulates cellular proliferation of the ovary. It has previously been demonstrated that estradiol can stimulate ovary cells to proliferate (including granulosa and theca cells) (Rao *et al.*, 1978); phenolphthalein may mimic the action of this steroid hormone by interacting with estrogen receptors of ovary cells. Alternatively, phenolphthalein may act as an antiestrogen in the pituitary gland and lead to an increase in gonadotropin production.

No chemical-related ovarian tumors have been reported in rats in any of the NTP studies. In the phenolphthalein study in rats, there was no tumor response at this site. Therefore, in our model systems, the mouse is more sensitive to a chemical-induced ovarian tumor response than the rat.

Cancer of the ovary is the fourth most common cancer in American women. There were an estimated 26,600 new cases diagnosed in 1995 and 14,500 deaths from this disease (SEER, 1995). In humans, approximately 80% of the ovarian tumors are of epithelial origin; other ovarian tumors may be of sex-cord stromal origin. Like many cancers, ovarian cancer is a disease of aging, with almost half of new cases occurring in women age 65 or older (SEER, 1995). In these mouse studies, ovarian effects were not observed in the 13-week study but developed toward the end of the 2-year study. The earliest occurrence of ovarian tumors in mice was noted on day 668. In this study, and in mice in general, the ovarian tumors were not fatal.

Environmental factors that might contribute to ovarian cancer in humans have not been identified. It is known that the rate for ovarian cancer in American women is two to three times higher than that for women in other parts of the world (Parkin *et al.*, 1993). It is not known if this difference in the ovarian cancer rate is due to environmental or genetic factors.

Like phenolphthalein, which has been shown to bind competitively to the estrogen receptor in MCF-7 cells (Ravdin *et al.*, 1987), other chemicals tested in the NTP bioassay have also been shown to interfere with estrogen binding at the estrogen receptor and to have mitogenic effects on breast cancer cells (Jobling *et al.*, 1995). These include zearalenone (NTP, 1982a), 2,4-dichlorophenol (NTP, 1989d), and butyl benzyl phthalate (NTP, 1982b, 1996b). However, of these chemicals, only phenolphthalein was shown to cause ovarian neoplasms in mice. Thus, demonstration of a chemical-induced estrogenic response (as reported from *in vitro* and/or *in vivo* studies) does not necessarily correlate with ovarian neoplasm or mammary gland neoplasm response in the F344/N rat and B6C3F$_1$ mouse studies. Further, these NTP chemicals with estrogenic activity show different neoplasm patterns in other tissues. This suggests that phenolphthalein-induced neoplasms may be due to multiple factors, which might include specific metabolism and distribution of the chemical, estrogenic effects of the chemical, or genotoxic properties of the chemical.

Estrogenic activity is found with compounds having different chemical structures, including estradiol, zearalenone, coumestrol, *o,p'*-DDT, kepone, bisphenol A, and impurities in phenol red (Katzenellenbogen, 1995). More information is needed on the molecular details of the three-dimensional structure of the estrogen receptor and its interaction with ligands to understand how these varied chemicals interact with the receptor. Studies with phenol red (phenolsulfonphthalein) have shown that lipophilic impurities in this dye have more estrogenic activity than the parent compound (Bindal and Katzenellenbogen, 1988; Bindal *et al.*, 1988), suggesting that the lipophilicity of chemicals is an important property in reaching or binding to the estrogen receptor.

The incidences of germinal epithelial degeneration of the testis in all exposed groups of male mice were significantly greater than that in the controls. The lesion varied from involvement of a few to all seminiferous tubules. Within the tubules, there was variable loss of sperm and germinal epithelial cells

(spermatogonia, spermatocytes, and spermatids), often with only Sertoli cells remaining. In the most severe cases, the cross-sectional diameter of the testis was decreased. Although more severe, this lesion is consistent with that observed in the 13-week study.

There was an increased incidence of myelofibrosis of the bone marrow of 12,000 ppm male and female mice and an increased severity of this lesion in exposed female mice. This was characterized by focal to multifocal replacement of bone marrow elements by clusters of loosely arranged spindle cells. The finding that phenolphthalein affects the bone marrow cells of mice in both the 13-week and 2-year studies shows that, in both short-term and long-term exposures, the bone marrow is a target tissue. Fibro-osseous lesions are observed in the flat and long bones of a number of strains of mice as spontaneous and induced lesions, and these lesions are very common in female $B6C3F_1$ mice. Female sex hormones, especially estradiol, appear to play a major role in the spontaneous development of myelofibrosis. Estrogen has been reported to cause neutropenia, thrombocytopenia, decreased bone marrow cellularity, and increased incidences of splenic hematopoiesis in mice (Fried *et al.*, 1974; Adler and Trobaugh, 1978). Witt *et al.* (1995) have also shown that phenolphthalein can induce micronuclei in bone marrow erythrocytes of mice. Micronuclei are formed from acentric chromosomal fragments or whole chromosomes generated through a variety of mechanisms such as mitotic loss of acentric fragments, mechanical consequences of chromosomal breakage and exchange events resulting in abnormal anaphase separation, or mitotic loss of whole chromosomes due to centromere or spindle failures. Because phenolphthalein induced chromosomal aberrations in cultured Chinese hamster ovary cells, induction of chromosomal breakage may be one of the mechanisms for the observed alterations in mouse bone marrow following exposure to phenolphthalein.

The decreases in the incidences of liver neoplasms and nonneoplastic lesions and the decreases in the incidences of proliferative lesions of the pituitary gland in female mice, the thyroid gland in female mice, and the tooth of male mice were considered to be chemical related.

There was no indication that phenolphthalein caused an increase in the incidences of lesions of the colon or rectum in rats or mice. In one limited study conducted in Australia to identify risk factors for colorectal cancer in humans, there was no indication that phenolphthalein taken as a laxative caused any disease in the colon or rectum (Kune, 1993). This study did not examine the potential of phenolphthalein to cause toxic or carcinogenic effects at other sites.

After oral administration, phenolphthalein is absorbed by the small intestine and is conjugated in the liver to phenolphthalein glucuronide, which passes into the colon where it is deconjugated and the active compound, phenolphthalein, is released. In humans, the laxative response may occur after an oral dose of phenolphthalein or the phenolphthalein glucuronide (Anand *et al.*, 1994).

In the 2-year studies, the plasma concentrations of total phenolphthalein were similar for all exposure concentrations. There was no consistent difference in plasma concentrations of total phenolphthalein between males and females. The findings that the plasma concentrations of total phenolphthalein were approximately the same (approximately 100 to 200 μg/mL plasma in rats and mice) for the exposure concentrations used in the 2-year studies may explain why dose-response increases in the incidences of kidney neoplasms in male rats, histiocytic sarcoma in male and female mice, malignant lymphoma in female mice, or ovarian neoplasms in female mice did not occur. However, in the single-dose toxicokinetic studies of phenolphthalein, the elimination half-lives in mice were approximately half those of rats (Appendix O; NTP, 1994).

The use of phenolphthalein as a laxative began early in the twentieth century (von Vámossy, 1908), and systematic studies of plasma concentrations of phenolphthalein or its metabolites in humans have not been reported in the literature.

Phenolphthalein was negative in the *Salmonella* mutagenicity assay with and without metabolic activation, but was positive in an *in vitro* chromosomal aberrations test conducted with S9 metabolic

activation. In conjunction with the 13-week feed study in mice, blood samples were analyzed at the end of study for the presence of micronucleated erythrocytes, and these were found to be significantly increased in male and female mice at exposure concentrations of 6,000 ppm phenolphthalein or higher (Witt *et al.*, 1995). In feed studies in Swiss (CD-1®) mice, phenolphthalein was found to increase the frequency of micronucleated erythrocytes in mice receiving 1,000 ppm phenolphthalein in feed for 14 weeks (Witt *et al.*, 1995).

Metabolism appears to be needed for genotoxicity with phenolphthalein. This hypothesis is supported by the requirement for S9 metabolic activation enzymes for the positive response in the chromosomal aberrations test in cultured Chinese hamster ovary cells. However, the predominant type of chromosomal damage observed in cultured Chinese hamster ovary cells treated with phenolphthalein was X chromosome breakage, a phenomenon that is not well understood, has been noted with only a few chemicals, and is only observed in cell cultures treated in the presence of S9. Therefore, the significance of the cultured Chinese hamster ovary cell results to interpretation of the *in vivo* responses is unclear. It is possible that the genetic damage induced by phenolphthalein is limited to chromosomal breakage (phenolphthalein, if converted to a quinoid, induces free oxygen radicals) and that point mutations and/or small deletions, such as are detected in the *Salmonella* assay, as well as other types of genetic damage, are not induced. For example, phenolphthalein did not induce sister chromatid exchanges in cultured Chinese hamster ovary cells with or without S9. It is unusual for a chemical to induce chromosomal aberrations but not sister chromatid exchanges in cultured Chinese hamster ovary cells, and this pattern of activity further supports the specificity of genetic damage induced by phenolphthalein.

In summary, phenolphthalein caused toxicity to the reproductive system of male and female mice. Phenolphthalein also caused neoplasms and nonneoplastic lesions in various tissues and organs in rats and mice (Tables 24 and 25). The mechanisms for these effects are not known; however, phenolphthalein has been shown to have estrogenic activity *in vitro* (Ravdin *et al.*, 1987) and *in vivo* (Nieto *et al.*, 1990) and genotoxic activity *in vivo* (Witt *et al.*, 1995). As is shown in Figure 1, phenolphthalein is capable of being converted to a quinoid structure. Quinoids are highly reactive chemicals capable of reacting with sulfhydryl groups and amino groups and forming oxygen free radicals (Brunmark and Cadenas, 1989; Degen and Metzler, 1989; Thompson *et al.*, 1990). It is not known at what specific sites in the cell these reactions might occur. Specific microenvironment conditions (e.g., pH) might be conducive to the formation of the phenolphthalein quinoid. Multiple mechanisms may be involved in causing the toxic/carcinogenic responses to phenolphthalein in the rodent.

CONCLUSIONS

Under the conditions of these 2-year feed studies, there was *clear evidence of carcinogenic activity** of phenolphthalein in male F344/N rats based on markedly increased incidences of benign pheochromocytomas of the adrenal medulla and of renal tubule adenomas and adenomas or carcinomas (combined). There was *some evidence of carcinogenic activity* of phenolphthalein in female F344/N rats based on the increased incidences of benign pheochromocytomas of the adrenal medulla in the 12,000 ppm group and of benign or malignant pheochromocytomas (combined) in the 12,000 and 25,000 ppm groups. There was *clear evidence of carcinogenic activity* of phenolphthalein in male B6C3F$_1$ mice based on increased incidences of histiocytic sarcomas and of malignant lymphomas of thymic origin. There was *clear evidence of carcinogenic activity* of phenolphthalein in female B6C3F$_1$ mice based on increased incidences of histiocytic sarcomas, malignant lymphomas of all types, lymphomas of thymic origin, and benign sex-cord stromal tumors of the ovary.

Exposure of rats to phenolphthalein in feed for 2 years resulted in increased incidences of focal hyperplasia of the adrenal medulla in males and in increased incidences and/or severity of nephropathy of the kidney in males and females. Exposure of mice to phenolphthalein in feed for 2 years resulted

in increased incidences of atypical hyperplasia of the thymus in males and females, degeneration of the germinal epithelium of the testis in males, and ovarian hyperplasia in females.

Exposure of mice to phenolphthalein in feed for 2 years resulted in decreased incidences of hepatocellular neoplasms and nonneoplastic lesions in males and females.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 12. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 14.

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APPENDIX B

Description of Online Searches for Phenolphthalein

DESCRIPTION OF ONLINE SEARCHES FOR PHENOLPHTHALEIN

Searches were limited to 1995 [the year before the NTP bioassay (NTP, 1996) which has an extensive literature review] through July 1997.

Online searches for phenolphthalein [CASRN 77-09-8] were performed in databases on the systems of STN International, DIALOG, NLM's TOXNET, and the Chemical Information Systems from 1980 to date. Toxicology information was sought in the EMIC, EMICBACK, RTECS, TOXLINE, CANCERLIT, EMBASE, BIOSIS, and MEDLINE (name and CASRNs combined with terms of metabolism and the MESH heading for all neoplasms). Occupational safety and health information was obtained from NIOSHTIC. HSDB provided a general review. The Chemical Abstracts Service Registry file and SANSS provided chemical identification information.

Market information, including production, shipments, sales and consumption, labor use, and workers by type was sought in PROMT (The Predicasts Overview of Markets and Technology) and The Chemical Economics Handbook database.

Structural analogs were also searched including, picosulfate [10040-45-6], oxyphenisatin [125-13-3], phenolphthalein diacetate [115-33-3], phenolphthalein disulfate [37811-54-4]. sulisatin [54935-03-4], deacetylbisacodyl [603-41-8], phenol red [143-74-8], bisacodyl [603-50-9].

Regulatory information was obtained from the latest quarterly update of the in-house FESA CD-ROM containing the latest *Code of Federal Regulations* and the *Federal Register* pertaining to the titles 21 (FDA), 29 (OSHA), and 40 (EPA) regulations. Updates on FDA regulatory actions were sought in the Federal Register full text database and in DIOGENES as well as at the FDA World Wide Web site on the Internet.

Also, the review of 1200 life sciences journals was accomplished using Current Contents on Diskette® for current awareness.

APPENDIX C

Report on Carcinogens (RoC), 9th Edition
Review Summary

Report on Carcinogens (RoC), 9th Edition
Review Summary

Phenolphthalein

NOMINATION
Review based on results of an NTP Bioassay of Phenolphthalein (1996), reporting clear evidence of carcinogenicity in 3 of 4 experimental animal groups.

DISCUSSION
Phenolphthalein is used as a laboratory reagent and acid-base indicator and has been used as a cathartic drug in over-the-counter laxative preparations. Experimental animal cancer results indicate clear evidence of benign and malignant tumor formation at multiple tissue sites in multiple species. Phenolphthalein causes oxidative stress and also demonstrates the capability to alter p53 pathways; both are considered common mechanisms involved in human cancers. The recommendations from the three NTP reviews of this nomination are as follows:

Review Committee	Recommendation	Vote
NIEHS (RG1)	list as a reasonably anticipated human carcinogen	9 yes/1 no
NTP EC Working Group (RG2)	list as a reasonably anticipated human carcinogen	7 yes/0 no/1 a*
NTP Board RoC Subcommittee	list as a reasonably anticipated human carcinogen	6 yes/0 no

*a-abstentions

Public Comments Received
A total of 3 public comments were received, all providing comments on the content of the background document prepared for the review of this nomination.